

THE USE OF PHOSPHORUS-BASED REAGENTS IN THE SYNTHESIS OF
SUBSTANCE P ANALOGUES

by

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To my parents

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COURSES ATTENDED

During my time in the department at Edinburgh University I have attended the following courses;

Organic Departmental Seminars, Various Lecturers.

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Application of X-ray, Crystallography Various Lecturers.

Research in the Pharmaceutical Industry, Various Lecturers.

ABSTRACT

Diphenylphosphinyl chloride (DppCl) was used as a coupling reagent in the synthesis of Met⁵-enkephalin by a modified phosphinic-carboxylic mixed anhydride procedure. The use of DppCl and 1-oxo-1-chlorophospholane (CptCl) in peptide synthesis was further evaluated by the synthesis of analogues of Substance P. It has been shown that these reagents are compatible with the unmasked side-chains of methionine and tyrosine during the conditions of coupling and deprotection. An attempt was made to design substrates which might interact with the Substance P C-terminal amidating enzyme. None of the analogues exhibited any significant biological activity.

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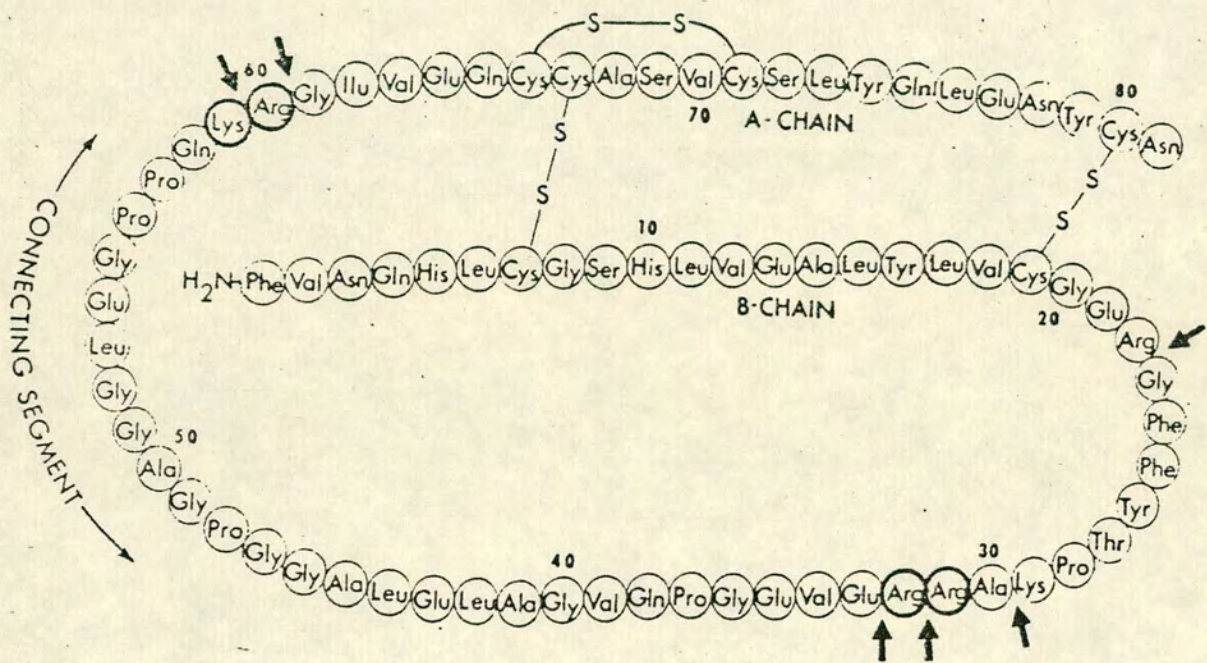


Fig.1.1

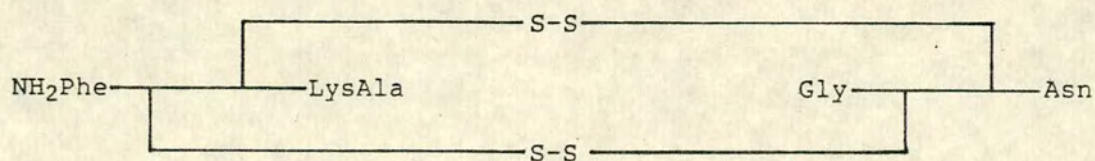
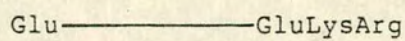
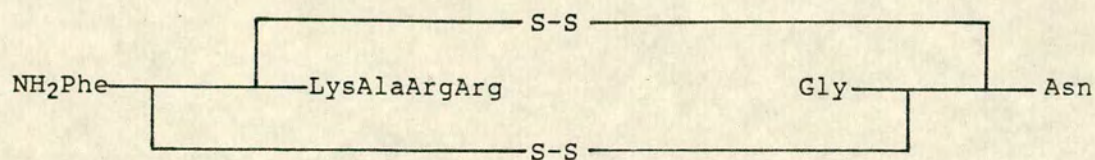
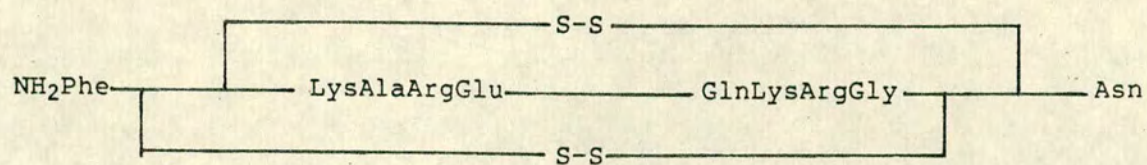
CHAPTER 1

INTRODUCTION

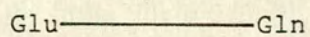
1.1. Processing of Peptide Hormones

From early studies on the isolation and characterisation of peptide hormones it became evident that they coexisted with larger polypeptides both in the glands of origin and in the circulation systems¹. Some of these polypeptides were shown to be precursors of the corresponding hormones and have been isolated and fully characterised. They have been found to have a much higher molecular weight. Sequencing of the precursors has shown them to contain several peptide hormone sequences, flanked on either side by a pair of basic amino acid residues, usually arginine or lysine (Fig.1.1). It is thought that the processing of the hormone is started by proteolytic cleavage with a trypsin-like enzyme, followed by removal of the C-terminal basic residues with an enzyme with specificity similar to carboxypeptidase B.

One of the first precursors to be identified was proinsulin (1), and its conversion to insulin (2) has been carefully studied.² Incubation of proinsulin with trypsin produces insulin with two additional arginyl residues on one of the C-termini. Prolonged incubation gives rise to cleavage of Arg-Arg bond as well as Lys-Ala bond in positions 29-30 of the B chain. So, although trypsin cleaves some of the bonds required for the formation of insulin, it does not give rise to the correct products. Isolation and sequencing of the C chain from pancreases of several mammalian species shows that the major product is devoid of the basic C-terminal amino acids originally present in proinsulin. Hence a second protease, with carboxypeptidase B like specificity, is present or the processing enzyme must be a novel proteolytic enzyme having unprecedented specificity which allows it to cleave at neutral as well as basic



Insulin (2)



C-peptide (3)

Fig.1.2

sites in proinsulin. Mixtures of trypsin and carboxypeptidase B have been incubated with proinsulin and major products isolated were insulin and the C-peptide fragment (3). These *in vitro* model studies strongly suggest that the *in vivo* converting system may work in a similar fashion. The overall conversion is shown below(Fig.2.2).

In various other systems precursors of hormones have been isolated and characterised and found to have the peptide hormone sequence connected to the remainder of the peptide chain by a pair of basic amino acids. The prohormone of the parathyroid hormone (4) has been identified and characterised ³ and found to be the same as a larger active peptide called "Calcemic Fraction A" earlier isolated by Hamilton and coworkers ⁴. The precursor is made up of the 86 residue parathyroid hormone with additional peptide material linked to its N-terminus via a basic residue. Another example is the 90 residue sheep pituitary peptide β -lipotropin (5) which contains the amino acid sequence of sheep β -melanocyte-stimulating hormone (6) (β MSH) residues 41-58, linked to the remainder of the polypeptide chain at either end through a pair of basic amino acids.

1.2. C-Terminal Amide Formation

A C-terminal amide functionality has been found in a range of peptides varying in size from the three residue thyrotropin-releasing hormone (7) to the 39-residue growth hormone-releasing hormone (8), and within this range >50% of the known peptide hormones are amidated. The amide functionality is often essential for biological activity since analogues possessing a C-terminal carboxyl group show greatly reduced activity. Also the amide functionality can protect, to a certain degree, the peptide from degradation by carboxypeptidases. Although the C-terminal residue of the peptide may vary,

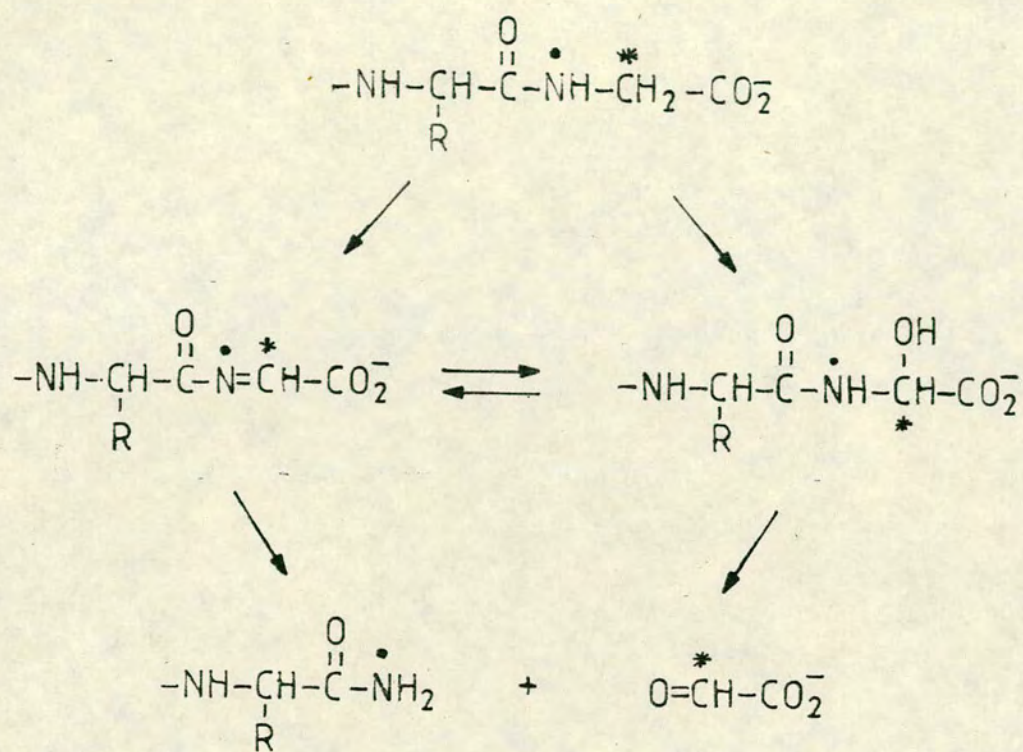


Fig.1.3

for example oxytocin (9), vasopressin (10) and luliberin (11) have a glycnamide, calcitonin (12) and thyroliberin (13) have a prolinamide, secretin (14) and α -melanotropin (15) end in valinamide and gastrin (16) terminates in phenylalanine amide, those whose precursors are known all have a glycine residue following the peptide fragment (for example the precursors of α -melanotropin,⁵ and the putative precursors of vasopressin⁶ and calcitonin^{7,8}). Since many of these peptides have little or nothing in common other than possessing a C-terminal amide functionality and a glycine residue in the same relative position in their precursors it was thought that the glycine was involved as a signal to processing amidating enzyme.

Bradbury and Smyth have managed to isolate and partially purify an amidating enzyme from the porcine pituitary⁹. Ammonium sulphate precipitated extracts from porcine homogenates were subjected to gel filtration. A broad fraction with amidating activity was collected, which suggested a mixture of enzymes with different molecular weights or aggregates of a single enzyme. Attempts at further purification using ion-exchange chromatography failed due to the instability of the enzyme. Recovery of amidating activity varied from 10-50% and this was thought to be because of the removal of cofactors, which would lead to denaturation of the enzyme. Thus, for amidation experiments, the enzyme was only partially purified, by gel filtration, and was known to contain other enzymes such as peptidases.

Treatment of the model tripeptide D-TyrValGly (17) with porcine homogenates or chromatographed preparations of the amidating enzyme gave the corresponding dipeptide amide D-TyrValNH₂ (18). The ValGly sequence was chosen because this was the minimum structural element of corticotropin (19) necessary for the formation of the valine amide group present at the

C-terminus of α -melanotropin. Due to the presence of aminopeptidases in the tissue homogenates the D isomer of tyrosine was chosen for the amino terminal residue to prevent degradation. Also the tyrosine could be radio-iodinated which was necessary for the assay. The reaction was monitored by subjecting aliquots of the reaction mixture to Sephadex SP ion exchange chromatography and measuring the radioactivity of the tripeptide and dipeptide amide fractions. The overall reaction was found to proceed in a maximum yield of 38% in 17hrs. Longer times led to lower yields which suggests that the dipeptide amide is being destroyed by other enzymes present in the pituitary extract.

A mechanism was proposed from the results obtained from subjecting isotopically labelled tripeptides to the amidating enzyme and analysis of the mass spectra of the purified derivatised products. Hence D-TyrValGly was made with both ^{14}N and ^{15}N incorporated into the valyl glycine peptide bond and the corresponding D-TyrValNH₂ was isolated as before. The low resolution mass spectra of the trifluoroacetyl derivatives⁹ of the dipeptides gave three ions which showed an increment of one mass unit expected from the incorporation of ^{15}N isotope. These mass spectra were identical to low resolution mass spectra of synthetic dipeptide amides, molecular ion 549 and 550 atomic mass units (amu), base peak 346 and 347 amu. High resolution mass measurements of molecular ions confirmed the empirical formulae of the isolated compounds. From these results the suggestion that the amide group might be derived from ammonia either by direct amidation of the valine carboxyl group¹⁰ or by transamidation of the valyl glycine peptide bond¹¹ can now be ruled out. The data supports the mechanism that involves the removal of hydrogen from the C-terminal glycine or its replacement by a hydroxyl group followed by cleavage of the C-N link (Fig.1.3). A further experiment to support this

Table I

Effect of Some Enzyme Inhibitors on the Amidating Enzyme from Porcine Pituitary

Reagent	Concentration	Effect
Mercaptoethanol	1 mM	—
	10 mM	30% Inhibition
Iodoacetamide	1 mM	—
PMSF	0.1 mM	—
Trypsin inhibitor	5 µg/ml	—
Benzethonium chloride	0.1 mM	—
Bacitracin	0.1 mg/ml	—
Pepstatin	5 µg/ml	—

mechanism involved the use of dTyrValGly made with ^{14}C -glycine. The isotope was found to be incorporated into the glyoxylate byproduct. Also the amidation reaction was totally unaffected when carried out in the presence of 1mM ammonium ion, glutamine or asparagine.

Studies into the properties of the amidating enzyme showed that its activity was not enhanced by the addition of cofactors which have been known to be associated with oxidative enzymes (such as NAD,NADH,NADP,NADPH). However, when the enzyme was dialysed against ethylenediaminetetraacetic acid all activity was lost indicating the possibility that the enzyme is associated with a metal ion. Copper II ions were found to restore full enzymic activity and cofactors such as ascorbate or catecholamine enhanced this activity. This was interesting since hydroxylase enzymes, which also contain copper II ions , use catecholamines and ascorbate as cofactors. No decrease in amidating activity was observed when specific enzyme inhibitors were used in order to gain information on which functional groups were important for amidating activity and it was found that there are no sulphydryl, disulphide or serine residues near the active site (Table 1.1). All these inhibitors have a potent effect on enzymes such as trypsin, chymotrypsin and pepsin, which are similar in nature to the proteolytic enzymes, and so could be included in the early stages of the purification of the amidating enzyme reducing the proteolytic degradation of it by the contaminating enzymes. Finally the presence of oxygen as opposed to air, has been shown to increase the rate of amidation three-fold. Although the mechanism is not known it is not surprising that molecular oxygen is involved, with copper II ions as cofactors, to produce the glyoxylate.

When studying the specificity of the pituitary enzyme several interesting facts arose. Glycine was found essential as the C-terminal residue for

amidation to occur⁹. Substitution of leucine, alanine, sarcosine, lysine or glutamic acid did not give any detectable dipeptide amides. Hence the Tyr-Val linkage of D-TyrValCOOH (20) would not be expected to break to form the D-TyrNH_2 (21) and D-TyrValCOOH (20) can be ruled out as an intermediate in the formation of D-TyrValNH_2 (18). Substitution in position 2 of the tripeptide with phenylalanine or glycine gave, upon treatment with the amidating enzyme, the corresponding dipeptide amide at a similar rate to that of D-TyrValGly ¹³. Therefore the pituitary enzyme could be capable of catalysing the formation of gastrin, oxytocin and vasopressin as well as hormones terminating in valine amides such as α -melanotropin. When position 2 was substituted with an acidic residue, for example glutamic acid, or a basic residue, such as lysine, the rate of conversion to the amide was significantly reduced. The optimum pH of the reaction mixture was found to be 6.7. If histidine is used in position 2 the reaction proceeds best at pH7, although not as quickly as with the neutral peptides. These results suggest the pituitary enzyme prefers a neutral substrate. To further study the specificity, tripeptide analogues of D-TyrPheGly (22) with neutral D-amino acids in the C-terminal position were subjected to the pituitary enzyme¹⁴. Initial competition experiments showed that peptides ending in D-Ser or D-Leu did not affect the rate of formation of $^{125}\text{I D-TyrPheNH}_2$ from $^{125}\text{I D-TyrPheGly}$. However, addition of D-TyrPheGly (23) or D-TyrPheDAla (24) significantly reduced the rate of formation of $^{125}\text{I D-TyrPheNH}_2$. D-TyrPheGly was found to have a larger effect than D-TyrPheDAla . Treatment of the pituitary enzyme with the four radio-iodinated labelled tripeptides mentioned above revealed as expected the analogues ending in D-Ser or D-Leu did not undergo amidation whereas the analogues terminating in DAla or Gly gave high yields of $^{125}\text{I D-TyrPheNH}_2$. It was notable that the amidation of $^{125}\text{I D-TyrPheGly}$ was at a much higher rate than $^{125}\text{I D-TyrPheDAla}$. Thus from these results a peptide

terminating in DAla can be used as a substrate for the amidating enzyme, although a peptide ending in LAla cannot. The orientation of the substituent on the α carbon of the terminal residue is therefore important for enzyme-substrate interaction. The size of this substituent cannot be too big as in the peptides ending in D-Leucine or D-Serine where steric hindrance presumably prevents binding. For the amidation of peptides with a C-terminal glycine it would seem only one of the α -carbon hydrogen bonds is broken in the initial hydrogenolysis step. The enzyme-peptide interaction can also be affected by the penultimate residue where peptides with charged residues in position 2 are very poor substrates whereas neutral peptides are very good substrates. Interestingly no C-terminal amide peptide discovered to date has a charged residue in the C-terminal position. The pituitary enzyme must recognise the α -carbon-hydrogen bond, maybe the α -carbon-nitrogen bond of glycine or D-alanine and the nature of the penultimate residue which would suggest the enzyme has a highly compact substrate binding site .

So far all experiments have been carried out using an enzyme extract from the porcine or bovine pituitary. It has been shown that this enzyme can catalyse the amidation of substrates based on the C-terminal sequence of α -melanotropin and gastrin. Independently an enzyme extract from the bovine pituitary has been shown to convert pGlu-His-Pro-Gly (24) to pGlu-His-Pro-NH₂ (25) (thyrotropin releasing factor)¹⁵. A preparation on the rat thyroid, similar to that carried out for the extraction of the pituitary enzyme, yielded an amidating enzyme with similar properties to the pituitary enzyme. Conversion of DTyr-Val-Gly (17), DTyr-Phe-Gly (22) and DTyr-Pro-Gly (26) to the corresponding dipeptide amides by the porcine pituitary enzyme occurred at different rates with DTyr-Phe-Gly being the quickest and DTyr-Pro-Gly the slowest. Treatment of the thyroid extract with these three tripeptides yielded

the corresponding dipeptide amides at the same relative rates. This is very interesting since if the amidating enzyme in the thyroid gland was specific for the thyroid hormone, calcitonin, which terminates in proline amide, the thyroid enzyme might be expected to catalyse the amidation of D-Tyr-Pro-Gly faster than D-Tyr-Val-Gly since the latter is a model substrate for the pituitary peptide, α -melanotropin. Similarly the pituitary enzyme would be expected to catalyse the amidation of valine or glycine containing substrates, reflecting the C-terminal sequence of α MSH or oxytocin and vasopressin, faster than D-Tyr-Pro-Gly. However, the amidation of the substrates D-Tyr-Pro-Gly, D-Tyr-Val-Gly and D-Tyr-Phe-Gly by the amidating enzyme exhibit relative reaction rates that are unrelated to the tissue of origin.

Since the small peptide D-Tyr-Val-Gly could be amidated it was interesting to note the minimum length required for amidation by the enzyme. Using competition experiments, very similar to those previously described, glycylglycine did not slow D-Tyr-ValNH₂ formation whereas acetylglycylglycine did. The interpretation of this was that the amidating enzyme did not accept NH₃⁺ groups of the glycylglycine in position 2. This has been seen before when analogues with charged residues in position 2 are not amidated by the enzyme. Glycylglycylglycine, which lacks the NH₃⁺ group in position 2, competed with D-Tyr-Val-Gly for amidation. In general the amidating enzyme only recognises two amino acids in the C-terminal region (neutral amino acids in position 2 and the glycine) which suggests the enzyme can amidate a variety of peptide hormones of varying size, such as the two mentioned previously. The problem with repeating *in vivo* amidations on large polypeptides *in vitro* is that the enzyme extract contains peptidases which compete with the amidation. Gastrinylglycine has been converted to gastrinamide using the amidating enzyme, purified by gel filtration but without the addition of cofactors, in a

30% yield. Purification and isolation of the amidating enzyme together with the use of suitable cofactors should improve this conversion enormously.

CHAPTER 2
THE ENKEPHALINS

TyrGlyGlyPheLeu

(28)

TyrGlyGlyPheMet

(29)

(30) α -endorphin: TyrGlyGlyPheMetThrSerGluLysSerGlnThrProLeuValThr.

(31) γ -endorphin: _____

(32) δ -endorphin: _____

(33) β -endorphin: _____

Leu.

PheLysAsnAlaIleIleLysAsnAlaTyr.

_____LysLysGlyGlu.

Fig.2.1

TyrGlyGlyPheMetLysLysMetAspGluLeuTyrProLeuGluValGluGluGluAlaAsnGly
GlyGluValLeuGlyLysArgTyrGlyGlyPheMet.

Peptide F (34)

SerProThrLeuGlnAspGluHisLysGluLeuGlnLysArgTyrGlyGlyPheMetArgArgVal
GlyArgProGluTrpTrpMetAspTyrGlnLysArgTyrGlyGlyPheLeu.

Peptide I (35)

TyrGlyGlyPheMetArgArgValGlyArgProGluTrpTrpMetAspTyrGlnLysArgTyrGly
GlyPheLeu.

Peptide E (36)

TyrGlyGlyPheMetArgArgValGltArgProGluTrpTrpMetAspTyrGlnLysArgTyrGly.

CHAPTER 2

THE ENKEPHALINS

2.1. Introduction

The enkephalins are two closely related pentapeptides which differ only in their C-terminal residue. They were first detected as an unknown factor which acted as an endogenous agonist of opiates in the human brain^{16,17} and soon characterised as low molecular weight peptides¹⁸. Mass spectra of the acetylated and methylated derivatives were originally used to sequence the peptides and showed them to be Leu⁵-enkephalin (28) and Met⁵-enkephalin (29)¹⁹. The sequences were confirmed by the isolation and characterisation of (28) and (29) from calf brain²⁰. As a result of discovering the enkephalins, and the fact that opioid receptors were known in the CNS, interest soon grew in the search for other opioid peptides. These compounds were thought to exist since it seemed highly unlikely that animals would have opiate receptors unless they already had an endogenous opiate system that interacted with opiate receptors in the absence of drugs.

Also the Met⁵-enkephalin sequence was found present in the 91amino acid pituitary polypeptide β -lipotropin (30)^{19,21,22}, which has no significant opioid activity, and similar pituitary peptides with morphinomimetic activity listed as α , β , γ and δ -endorphins (Fig.2.1). These latter peptides have primary structures corresponding to sub-units of β -lipotropin and as a result β -lipotropin was thought to be a precursor of Met⁵-enkephalin²².

2.2. Biosynthesis

It was originally thought that enkephalins, as with many other hormones and peptides, were derived from a larger precursor which is cleaved and degraded at the appropriate positions to give the product. If this is so

β -lipotropin and the endorphins are highly unlikely candidates as precursors. Apart from not explaining the biosynthesis of Leu⁵-enkephalin, the Thr-Ser sequence which links Met⁵-enkephalin to the rest of β -endorphin would be a very unusual cleavage site for enzymic production of the pentapeptide. Also, β -endorphin and the enkephalins have not been found in the same areas within the body. The enkephalins have been found in the posterior lobe of the pituitary whereas β -endorphin has been found only in the anterior and intermediate lobes of the pituitary²³, and tissues such as the striatum and adrenal medulla have been found to contain only the enkephalins in large concentrations but no β -endorphin^{24,25}. However, for conclusive evidence that β -endorphin is not involved in the biosynthetic pathway of enkephalins it would be necessary to isolate a precursor containing the enkephalins' sequences. Early work concentrated on searching for the precursor in the adrenal medullary chromaffin granules, which were known to contain large amounts of enkephalins and no β -endorphin²⁵. There are known to exist, within the bovine adrenal medulla, a number of polypeptides that bind to opiate receptors. The largest protein (>50,000 daltons) when treated with trypsin and carboxypeptidase B yields Met⁵-enkephalin and Leu⁵-enkephalin, in a ratio of 7:1²⁶. Other smaller polypeptides with molecular weights ranging from 500-22,000 daltons exist, from which Peptide F (34) and Peptide I (35) have been purified and their amino acid sequences determined²⁷. Peptide E (36) and BAM22P (37) are two smaller peptides which have since been isolated, purified and their amino acid sequences determined²⁸. Both of these latter peptides are partial sequences of peptide I (Peptide E is fragment(15-39) of Peptide I and BAM22P is fragment(1-22) of Peptide E). All these fragments together with the larger protein were all thought to be intermediates in the biosynthetic pathway of enkephalin. The production of enkephalins is thought to be similar to that of

other hormones since the enkephalin sequence within these peptides are flanked by two basic amino acids, thought to be signal sequences for the processing enzymes. This was originally challenged by the discovery of small enkephalin-size peptides two of which were Met⁵-enkephalin-Arg-Phe (38)²⁹ and Met⁵-enkephalin-Arg-Gly-Leu (39)³⁰. However, around about the same time, larger polypeptides were isolated and sequenced. In one of them, a 3,600 dalton peptide (40), at the C-terminal end was found Met⁵-enkephalin preceded by -Lys-Arg- and followed by -Arg-PheCOOH³¹. In the presence of the enkephalin processing enzyme the Met⁵-enkephalin-Arg-Phe sequence would be expected to be released which would explain its presence in chromaffin granules. Similarly a larger peptide (5,300 daltons) (41) was found to contain at its carboxyl terminus a -Lys-Arg- sequence followed by Met⁵-enkephalin-Arg-Gly-LeuCOOH³² which upon treatment with the processing enzyme would be expected to give Met⁵-enkephalin-Arg-Gly-LeuCOOH. With all this known it was interesting to note that a precursor (42) had been isolated from the bovine adrenal medulla using molecular cloning techniques and DNA sequencing to establish its amino acid sequence³³. The proenkephalin was found to contain six Met⁵-enkephalin and one Leu⁵-enkephalin sequences. Three of the Met⁵-enkephalin sequences were flanked with two basic amino acid sequences and two were preceded by a pair of basic amino acids and followed by Arg-Gly-Leu-Lys-Arg or Arg-PheCOOH. The size of the precursor was only 27,300 daltons and was believed to be a large fragment of the 50,000 dalton precursor originally observed. Since then proenkephalin originating from the adrenal medulla of human³⁴ and rat³⁵ has also been sequenced and found to be very similar differing only by one or two amino acids but all containing six Met⁵-enkephalin sequences (including the two extended enkephalins) and one Leu-enkephalin sequence.

(28) Leu-enkephalin	TyrGlyGlyPheLeu
(44) Leu-enkephalin-Arg ⁶	TyrGlyGlyPheLeuArg
(43) α -neo-endorphin	—————LysTyrProLys.
(45) β -neo-endorphin	—————.
(46) PH-8P	—————ArgIle.
(47) Dynorphin	—————ArgProLysLeuLys
	TrpAspAsnGln.

Fig.2.2

Extended Leu⁵-enkephalins have also been found within the hypothalamus, in the brain and in the pituitary. The first was called α -neo-endorphin(43)³⁶ and since then several others have been isolated, purified and their amino acid sequence determined³⁷(Fig.2.2).

Originally it was not fully understood how these extended Leu⁵-enkephalins were involved in the enkephalin biosynthesis until it was reported that the amino acid sequence of a precursor to β -neo-endorphin (45) and dynorphin (47) had been found³⁸. This precursor was derived from the porcine hypothalamus and found to contain the sequences of the above peptides plus Leu⁵-enkephalin itself. This prodynorphin precursor is also known as proenkephalin B whereas the proenkephalin is called proenkephalin A. These are obtained from different parts of the body and might not be connected. Studies are presently being undertaken to isolate the source of Met⁵-enkephalin in the brain and to see if there are any similarities with proenkephalin A from the adrenal medulla or if it is derived from a much bigger preproenkephalin containing proenkephalin A and B sequences, such as the original 50,000 dalton precursor found in the adrenal medulla.

2.3. Structure Activity Studies

In order to design super-agonists much structure activity work has been done on the enkephalins with the aim of finding the important functionality required for binding to the receptor site. From the earliest work blocking of the C-terminal carboxyl group by either dansylation or amidation³⁹ resulted in analogues with equal potency. However, nothing more than monomethylation could be done on the N-terminal amino group without losing biological activity. The tyrosine in position one was found to be essential. Any tampering with the aromaticity or the phenolic hydroxyl resulted in a complete loss of

I-TyrDAlaGlyPheLeu

(48)

I₂-TyrDAlaGlyPheLeu

(49)

activity^{39,40}. Mono-iodination of the tyrosine phenyl ring gave an analogue (48) with only 9–30% activity *in vitro* whereas the di-iodinated compound (49) had an exceedingly low potency⁴¹. This was interesting when considering radio-iodinated analogues for binding studies to receptor sites. A much better method would involve the C-terminal dansylated analogues using fluorescence for detection.

When early workers found the enkephalins to have very low activity in *in vivo* assays they soon realised that it was due to the breakdown of the pentapeptides by peptidases. Close examination of this degradation by thin layer chromatography showed the Tyr-Gly amide bond was cleaved. In an attempt to render this bond inaccessible to the cleavage enzymes Gly² was replaced by D-amino acids from which arose the analogue (50)⁴². This analogue was equipotent with Met⁵-enkephalin in receptor binding but is not degraded. Also it showed analgesic properties when injected intracerebroventricularly (icv) but no activity when administered intravenously (iv). Further work showed that analogues with either D-methionine or D-norleucine in position two were even more potent but analogues with basic residues (eg.D-lysine) or aromatic residues (eg.D-phenylalanine) in position two had diminished activity.

Modification in any way of the residues in positions 3 and 4 results in analogues with little or no activity.

Substitution of Leu⁵ or Met⁵ with norleucine results in analogues with comparable activity⁴³. This is interesting since it questions the need of the methionine sulphur atom or the β -branching of the leucyl side-chain. In an attempt to protect the amide bond between positions 4 and 5 Bajusz *et al* substituted proline for the terminal amino acid resulting in a significant

TyrDAlaGlyPheMet

(50)

TyrDMetGlyPhePro

(51)

TyrDAlaGlyPheNValNH₂

(52)

TyrDMetGlyPheNValNH₂

(53)

}

TyrDAlaPheMetNH₂

(55)

TyrDAlaPheLeuNH₂

(56)

increase in biological activity⁴⁴. Coupled with the results from other substitutions (51) evolved as a potent analogue which *in vivo* was 80x stronger than morphine⁴⁵ on icv administration and *in vitro* was up to 8x more potent than Met⁵-enkephalin. This increase in activity was put down to its stability towards peptidases and it assuming a different conformation to Met⁵-enkephalin resulting in favourable transport and or binding properties. At first this exceptional activity was thought to be due to the intracyclic amine functionality of the proline residue. However, analogues with norvaline in position 5 (52,53), where the proline ring is opened and the α -amino character is conserved, resulted generally in an increase in activity⁴⁶.

An early observation that Tyr-Gly-Gly-Phe (54) exhibited full enkephalin activity³⁶ led to a whole series of truncated enkephalins. A number of tetrapeptide analogues were reported to be potent in both *in vivo* and *in vitro* assays⁴⁷. (55) and (56) were equipotent with the parent pentapeptides on the GPI assay but less so on the rat tail flick test. An evaluation of the data led to a series of 25 tripeptides and 5 dipeptides being synthesised and assayed on the guinea pig ileum (GPI)⁴⁸. Tyr-Pro-Phe-NH₂ (57), Tyr-DAla-Phe-NH₂ (58), Tyr-DAla-Phe-CH₂OH (59) and Tyr-DPhe-Phe-CH₂OH (60) had 20-25% activity of Met⁵-enkephalin. Four aromatic alkyl amides of Tyr-DAla were shown to have full enkephalin-like activity on the GPI. Tyr-DAla-phenylpropyl amide(61) was found to possess 80% of the potency of Met⁵-enkephalin *in vitro* and is equipotent with Tyr-DAla-Gly-Met-NH₂ in producing analgesia upon icv injection.

2.4. Opiate Receptors

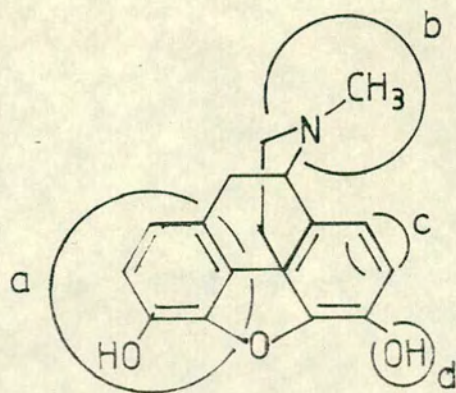
When it was first noted that some alkaloid opiates could not displace (³H)Tyr¹,Met⁵)enkephalin (62) from its receptor efficiently and enkephalin could

not compete for alkaloid binding sites⁴⁹ it was thought that this was due to different modes of binding to the same receptor or enkephalin induced conformational changes of the receptor⁵⁰. After further work it was suggested that this was due to multiple classes of opiate receptors with differing affinities for a given analogue^{51,52}.

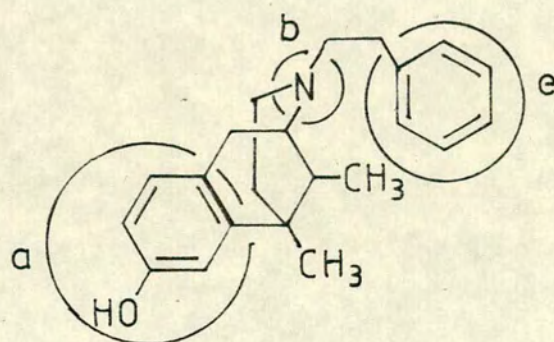
From the results of various bioassays and radioreceptor assays opiate drugs were found to be more potent in the GPI test than enkephalin where the reverse was true for the rat vas deferens assay⁵². Thus it was postulated that two types of receptors existed, a μ -receptor in the ileum and a δ -receptor in the vas deferens⁵³. When analogues with Gly² replaced by a D-amino acid were tested on both assays different activity profiles were obtained which is consistent with the idea of different classes of opiate receptor. Biological studies of recent analogues now consist of testing the compounds on both assays as in the case of (52) and (53) where it was also shown that (52) can discriminate between the two receptor types⁵⁴.

2.5. Conformation

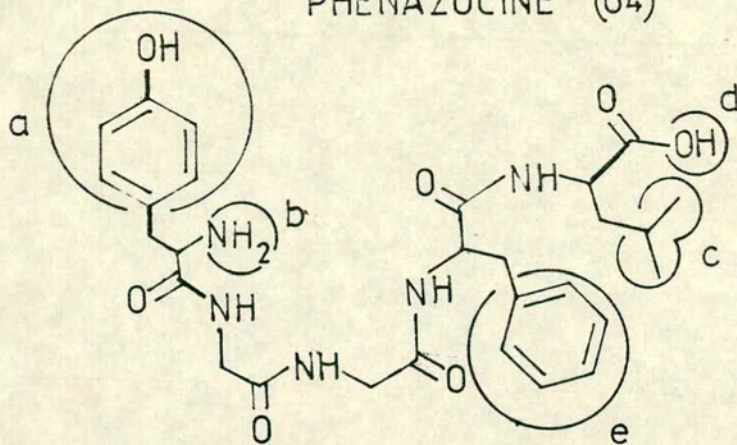
Since enkephalins are known to compete with drugs at opiate receptors it is reasonable to assume that the structural and conformational features of both the drugs and the enkephalins will be similar at the active site. The enkephalins are thought to have various conformations in solution and in a solid state based on theoretical minimum energy calculations⁵³ and proton⁵⁵ and carbon-13⁵⁶ magnetic resonance measurements and X-ray diffraction studies⁵⁷. The relevance of the results from these studies to the actual conformation at the active site is not yet known. Indeed from minimum energy calculations the preferred conformer of enkephalin does not resemble morphine(63)⁵³. Thus, in order to make a model of the structure of the enkephalins a mixture of physical



MORPHINE (63)



PHENAZOCINE (64)



Leu-ENKEPHALIN (28)

measurements and structure activity studies has been used to a limited extent. It has been shown that the tyrosine residue corresponds to the tyramine ring A moiety of morphine where both the hydroxyl group (equivalent to ring D hydroxyl) and the amino group (corresponding to the N-methyl group of morphine) of tyrosine are essential for activity. It is thought the tyrosine configuration may not have to be exact as long as the hydroxyl-amino distance remains fixed^{57,58}. This slight flexibility within the tyrosine could be induced by the two glycines which, through H-bonding, can act as a spacer arm and confer several orientations within the molecule. The exact conformation of the Phe and the Met (or Leu) is not fully understood except that the Phe is essential for biological activity. Maybe it corresponds to ring F found in some opiates such as phenazocine (64)⁵⁷, but not found in morphine. Hence opiate drugs may give an insight to the bioactive conformation of enkephalins but a greater knowledge of the receptor must be known before the picture is complete.

2.6. Discussion

A major reason for the success of much of the work done in the enkephalin field is because of the availability to the biologist of large amounts of Leu⁵- and Met⁵-enkephalin, as well as numerous analogues. As the knowledge of the role of enkephalins in living systems, especially in man, increases so does the need to be able to make them efficiently. Because of their short sequence and the synthetically agreeable amino acids which make up the enkephalins, these opioid peptides have been used as examples to test new methodology and reagents in peptide synthesis on numerous occasions, to the extent that there is a review on the subject⁵⁹. For our part the presence of tyrosine and methionine within the molecule presented an ideal opportunity to further evaluate the use of the diphenylphosphinyl group as an N- α -protecting group⁶⁰

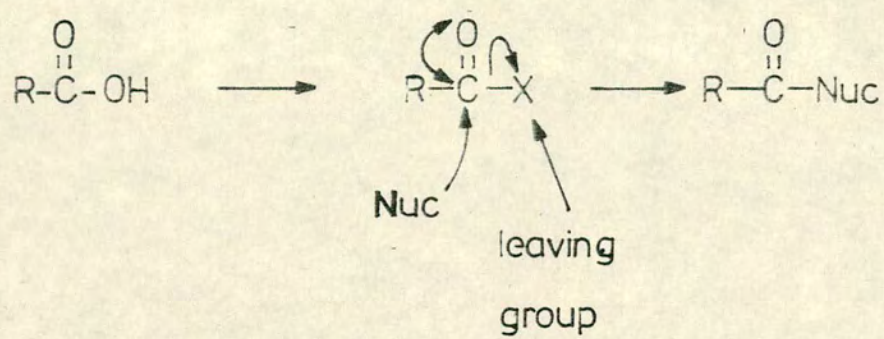


Fig.2.3

and in carboxyl activation *via* the carboxylic-phosphinic mixed anhydride method⁶¹.

2.6.1. Carboxyl Activation

The way in which a carboxyl group is activated towards nucleophiles is to attach a good leaving group to it which leaves the carboxyl function susceptible to attack by any electron rich species (Fig.2.3). Over the years this has been achieved in various ways by the use of active esters⁶², carbodiimides⁶³, symmetrical-⁶⁴ and mixed anhydrides⁶⁵. Of these methods mixed anhydrides result in the fastest acylation of amines. The reaction proceeds by the *in situ* formation of the mixed anhydride from the N- α -protected amino acid and the acid chloride at low temperatures ($< -20^{\circ}\text{C}$) under anhydrous conditions. The amino component is added, usually as the salt, and the mixture is stirred for 2–4 hours. The disadvantages of this method are:

1. The temperature must be kept below -20°C when forming the mixed anhydride or else disproportionation occurs.
2. Conditions must be scrupulously dry throughout the synthesis to obtain high yields otherwise the mixed anhydride readily hydrolyses to the starting amino acid
3. The nature of the R group is usually a bulky one to direct the incoming amino function to the appropriate carbonyl group. When the amino acid side chain R is also sterically bulky, such as in valine or isoleucine, the two groups are believed to crowd the approach of the nucleophile which can lead to much slower reactions and significant amounts of byproducts are formed derived from the attack of the amino function at the other carbonyl centre(Fig.2.4).

To overcome these problems the carboxylic-phosphinic mixed anhydride method was introduced to peptide synthesis⁶¹. There are a limited number of examples in the literature where the anhydride is derived from the phosphonic chloride (65) or the phosphonic azide (66) but there is speculation as to their

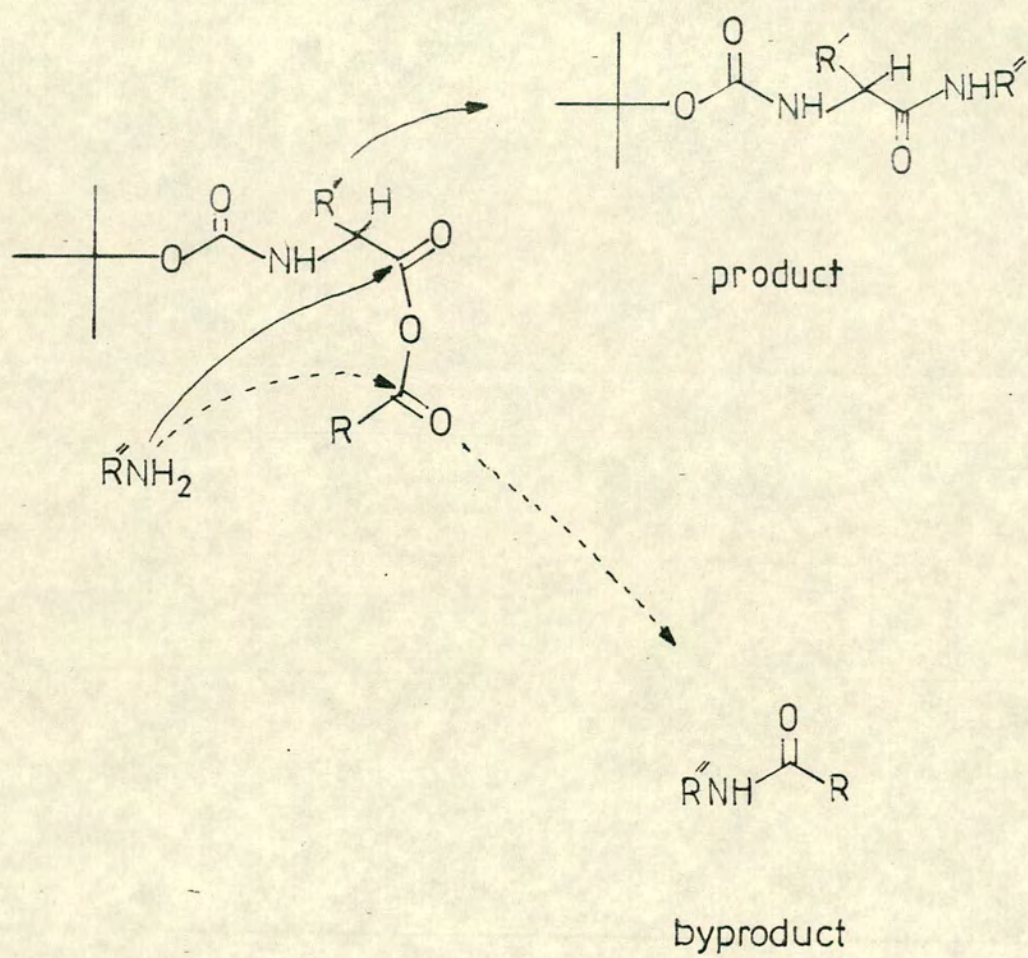
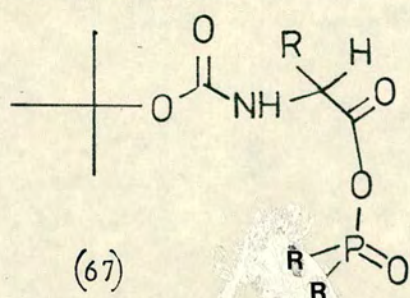


Fig.2.4



R = alkyl, Oalkyl or
aryl, Oaryl

exact mechanism of activation. Work studying the stability and reactivity of mixed anhydrides derived from phosphinic chlorides has led to the introduction of the diphenylphosphinic group (67) ($R=Ph, X=O$). ^{31}P nmr studies have shown the mixed anhydride to be formed instantaneously at $0^{\circ}C$. It is slow to react with water, or to disproportionate at room temperature, but reacts rapidly with a nucleophilic amine to form an amide bond. Furthermore no byproducts derived from the attack of the amino function at the phosphorus centre were found. This has been credited to the amino group's greater affinity for the carboxyl group rather than the phosphorus centre and the greater bond strength of the P-O bond over the C-O bond. A general procedure involves the formation of the mixed anhydride at $0^{\circ}C$ from the N- α -protected amino acid and diphenylphosphinyl chloride with one equivalent of base in methylene chloride. Although ^{31}P nmr shows the reaction to be instantaneous a period of 5-15 minutes is usually employed for activation. The amine component (often the methyl ester hydrochloride salt of an amino acid or peptide) is added in dimethylformamide (DMF) followed by one equivalent of N-methylmorpholine (NMM). In the reaction there is a 10% excess of the anhydride to the amine component. The amide bond formation is complete after stirring at $0^{\circ}C$ for one hour and room temperature for one hour. After a standard work up (see experimental) the peptide is isolated in a yield of approximately 80% after purification.

Attempts to increase the yield, by insuring totally dry conditions and the use of an inert dry atmosphere, failed to improve upon the yields that had been obtained before. Even though all the amine component was used up in the reaction, as seen on the tlc using ninhydrin for detection, and no byproducts were obtained after workup (except diphenylphosphinic acid (68) in the synthesis of Z-Gly-Gly-OMe) it was baffling as to why higher yields were not

PEPTIDE	% YIELD	% INCREASE
BocPheGlyOMe*	94	25
DppMetGlyGlyOMe	93	23
DppLeuGlyGlyOMe	83	14

Table.2.1

* Dr.S.Young; personal communication

obtained. Results obtained from the application of diphenylphosphinic mixed anhydrides to solid phase peptide synthesis⁶⁶, suggested that the diphenylphosphinic acid byproduct was forming a salt with the liberated amine component, thus removing the latter from the reaction. To counteract this one equivalent of base was added to the reaction mixture after the amine component and NMM had been added. The base used was 2,6-lutidine (2,6-dimethylpyridine) which was thought weak enough not to abstract an α -proton from the amino acid residues but strong enough to sequester any diphenylphosphinic acid. Racemization tests on model peptides have yet to be completed. An analogous situation to this has been reported in solid phase peptide synthesis where symmetrical anhydrides have been used for carboxyl activation. After acylation of the symmetrical anhydride the resulting unused amino acid forms a salt, in the same way as the diphenylphosphinic acid, with the liberated amino component. In this case diisopropylethylamine (69) was used as the base⁶⁷.

As a result of the addition of 2,6-lutidine yields of recrystallised dipeptides and tripeptides rose to 90–95% in many cases affording pure material (by tlc) (Table 2.1). This procedure was further evaluated by comparing the synthesis of Met⁵-enkephalin to an earlier preparation using the same conditions but without the extra base⁶⁸.

2.6.2. α -Amino and Carboxyl Protection

The most widely used protecting group for α -amino protection is based on the urethanes, $R-O-CO-$, where the nature of R can determine the conditions of cleavage. As a result a large number of urethane protecting groups are available which utilise a wide range of deprotecting conditions. Table 2.2 is a list of the more popular ones. However, as a result of acidolytic cleavage of

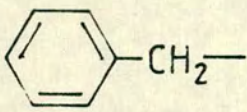
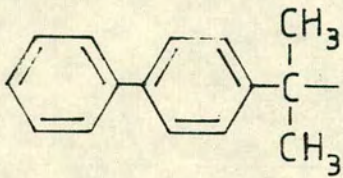
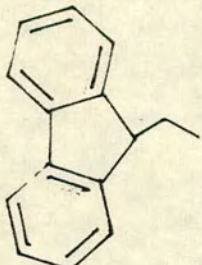
R	Cleavage Conditions	Lit.
	HBr/ AcOH	69
(CH ₃) ₃ C-	TFA	70
	AcOH	71
	20% piperidine	72

Table 2.2

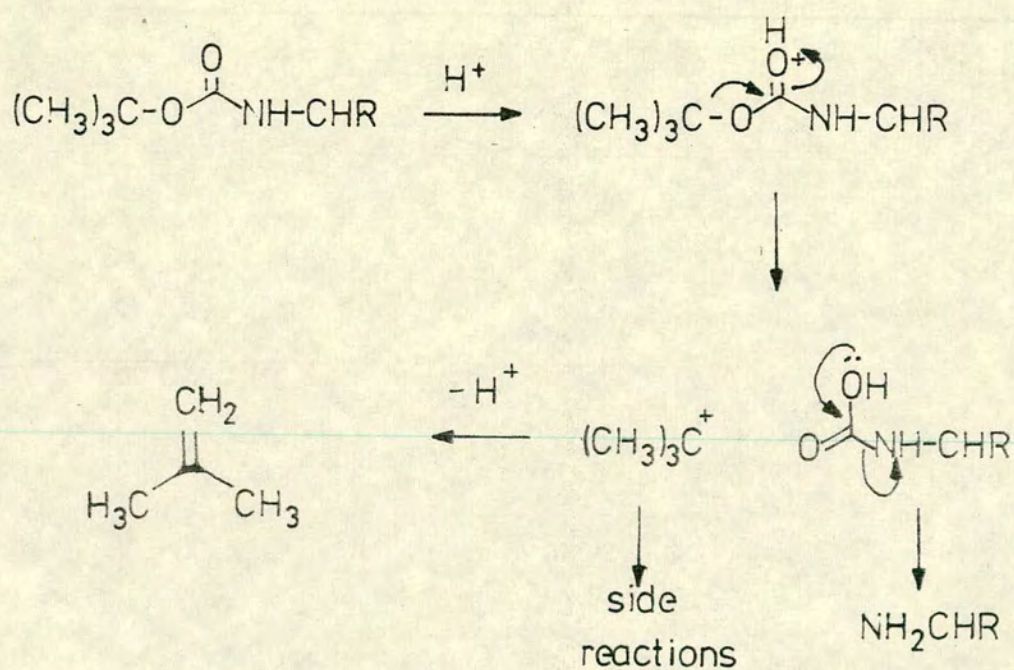
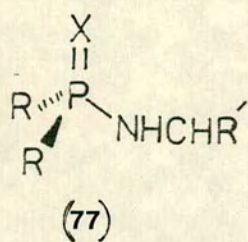


Fig.2.5

urethanes (which is the case for the majority of them) an intermediate carbenium ion is formed. While in some examples this species is very short lived, in others such as the benzyloxycarbonyl group (Z) (70) or the tertiary butyloxycarbonyl group (Boc) (71) the corresponding carbenium ion is relatively stable and can be involved in side reactions with certain amino acids with sensitive side chains, in preference to being converted to its breakdown products, in this case benzylbromide (72) and isobutylene (73) respectively (eg Fig.2.5). The side reactions involve alkylation of the side chains of tyrosine, tryptophan, methionine and cysteine. In the case of Boc group the corresponding tertiary butyl carbenium ion has been found to alkylate the indole moiety of tryptophan in three positions⁷³. With tyrosine, the phenolic hydroxyl group is alkylated if it is left unmasked. Therefore it is usually protected by etherification (see later). Model studies involving tyrosine in cleavage studies of tertiary butyl groups with trifluoroacetic acid have shown that the phenyl ring is butylated in the ortho position to the hydroxyl group in as much as 1% of the total reaction product⁷⁴. When using the Z group methionine has been found to be the most troublesome residue. Acidolytic cleavage using hydrogen bromide in acetic acid can lead to S-benylation of the sulphur, forming a sulphonium salt which decomposes to give the S-benzylhomocysteine derivative⁷⁵ in preference to reverting back to methionine. An alternative to acidic conditions was the use of sodium in liquid ammonia. Under these conditions the methionine can demethylate to form homocysteine⁷⁶. The sulphur functionality has also been found to poison palladium catalysts used in hydrogenolytic cleavage of the Z group. Although the product can be obtained using longer reaction times, the formation of α -aminobutyric acid has been reported to occur⁷⁷.

Even though these side reactions only occur in yields of 1–5%, repetitive



R=alkyl or aryl

X= O, S

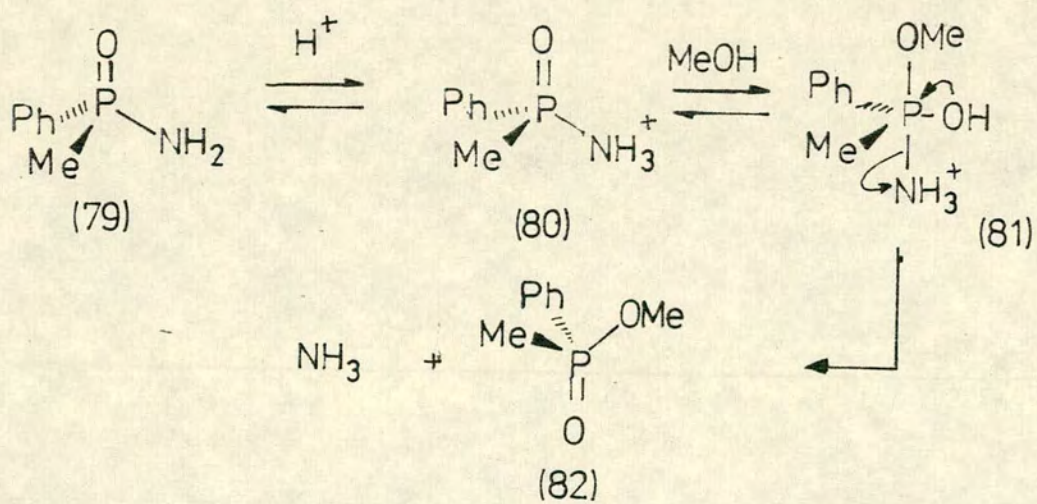
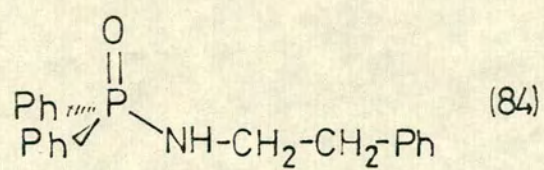
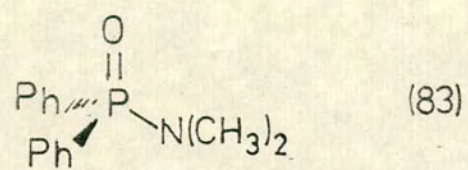


Fig.26

exposure of the sensitive amino acids, as occurs in a stepwise synthesis, can cause a serious lowering of the yield and an increase in impurities, making isolation of the final product much more difficult. For the stepwise synthesis of Met⁵-enkephalin the C-terminal methionine would be subjected to the deprotection conditions four times and a significant loss of product would be envisaged. Although scavengers such as anisole (74), thioanisole (75) or 1,2-dithioethane (76) can be used to remove the carbenium ions from the reaction mixture, the use of an acid labile protecting group which does not form any reactive intermediates during deprotection was thought to be more advantageous and, as a result of this protecting groups with a phosphinamide linkage to the amine component were introduced into peptide synthesis (77)^{78,60}. By varying the alkyl or aryl groups attached to the phosphorus the acid lability of the phosphinamide bond can be changed. When phenyl groups are attached to the phosphorus the resulting diphenylphosphinamide (78) can be cleaved by the use of mild acid. The mechanism is believed to be similar to that proposed by Harger for the cleavage of the chiral phosphinamide (79) using methanolic hydrogen chloride solutions⁷⁹ Initial protonation of the nitrogen is followed by nucleophilic attack at the phosphorus to form a trigonal bipyramidal intermediate (81) which dissociates to give the **phosphinate** (82) with no formation of any reactive intermediates and complete inversion at the phosphorus. From X-ray data on similar compounds (N,N-dimethyl-P,P-diphenylphosphinamide (83) and N-methyl-N-(2-phenylethyl)-P,P-diphenylphosphinamide (84)) the geometry of the nitrogen was found to be a flattened tetrahedron⁸⁰ with the nitrogen lone pair of electrons almost in the N-P-O plane whereas in the amide bond of the urethanes the trigonal nitrogen lone pair of electrons is orthogonal to the N-C-O plane. Hence the reduced delocalisation of the lone pair of electrons on the nitrogen with the



P=O bond results in the nitrogen of the phosphinic amide being much more basic than the nitrogen of the corresponding carboxylic amides.

The optimum deprotection conditions have been reported as six equivalents of hydrogen chloride in methanol at 35–40°C resulting in complete cleavage within twenty minutes⁶¹. This deblocking reaction was followed by tlc and ³¹P nmr spectroscopy. Alternative deprotection conditions were used for removing the final Dpp group since the C-terminal carboxyl group was deblocked and the use of methanolic hydrogen chloride solutions could lead to transesterification. Other options available include the use of trifluoroacetic acid in aprotic solvents, and acetic acid, formic acid mixtures. In conjunction with other work on the deprotection of the Dpp group using hydrogen chloride in trifluoroethanol it was decided to further evaluate the use of hydrogen chloride in dioxane, water mixtures for Dpp deprotection.

From the experience of earlier attempts at incorporating the phenyl ester function into the methionine residue⁶⁸ and the success of using the methyl ester for carboxyl protection when introducing the Dpp group⁶⁰ it was decided to use the methyl ester for C-terminal carboxyl protection. Originally the phenyl ester was to be used employing the mild alkaline peroxide conditions for a very quick deprotection. However, the introduction of the phenyl ester functionality to the methionine residue was fraught with difficulties and no efficient synthesis of MetOPh evolved. In contrast the synthesis of MetOMe as the hydrochloride salt is well established in the literature⁸¹. Also the use of hydrogen chloride in methanol for the deprotection step would not cause any transesterification problems. Removal of the methyl ester functionality is easily achieved with one equivalent of base at room temperature in 2–6 hours.

2.6.3. Side-Chain Protection

As previously mentioned, the two sensitive amino acids in the Met⁵-enkephalin sequence are methionine and tyrosine which are prone to side reactions during acidolytic cleavage of α -amino protecting groups.

Unlike most amino acids which require side-chain protection to eliminate byproduct formation this has been found not to be the case for methionine. The decision to use side chain protection is often taken by the workers after considering the nature, size and properties of the final peptide. If methionine is left unprotected the sulphur can be alkylated to form the sulphonium salt⁸² or oxidised to the sulfoxide⁸³[Met(O)]. Since not all S alkylation is reversible⁶⁴ this side reaction is often left to be corrected during the purification step. However, if methionine is used as the sulfoxide⁸⁴ the S-alkylation is eliminated because of the reduced nucleophilicity of the thioether side chain. Either way, there is always some uncertainty as to whether to convert the sulfoxide to methionine before or after purification. This problem has been somewhat alleviated when Met(O) is used in conjunction with other HF cleavable side-chain protecting groups by the use of a 1:3 mixture of HF and dimethylsulphide which will convert Met(O) to Met and cleave most benzyl-based protecting groups simultaneously⁸⁵.

By using the diphenylphosphinyl protecting group as the α -amino protecting group, which does not form any reactive intermediates during cleavage, the need for methionine protection is eliminated and so the thioether functionality was left unaltered during the Met⁵-enkephalin synthesis. In the work up step a stream of nitrogen bubbles was used to perturb the organic and aqueous layers instead of shaking. This was a precautionary step to eliminate the air oxidation of methionine. In all the syntheses to date no

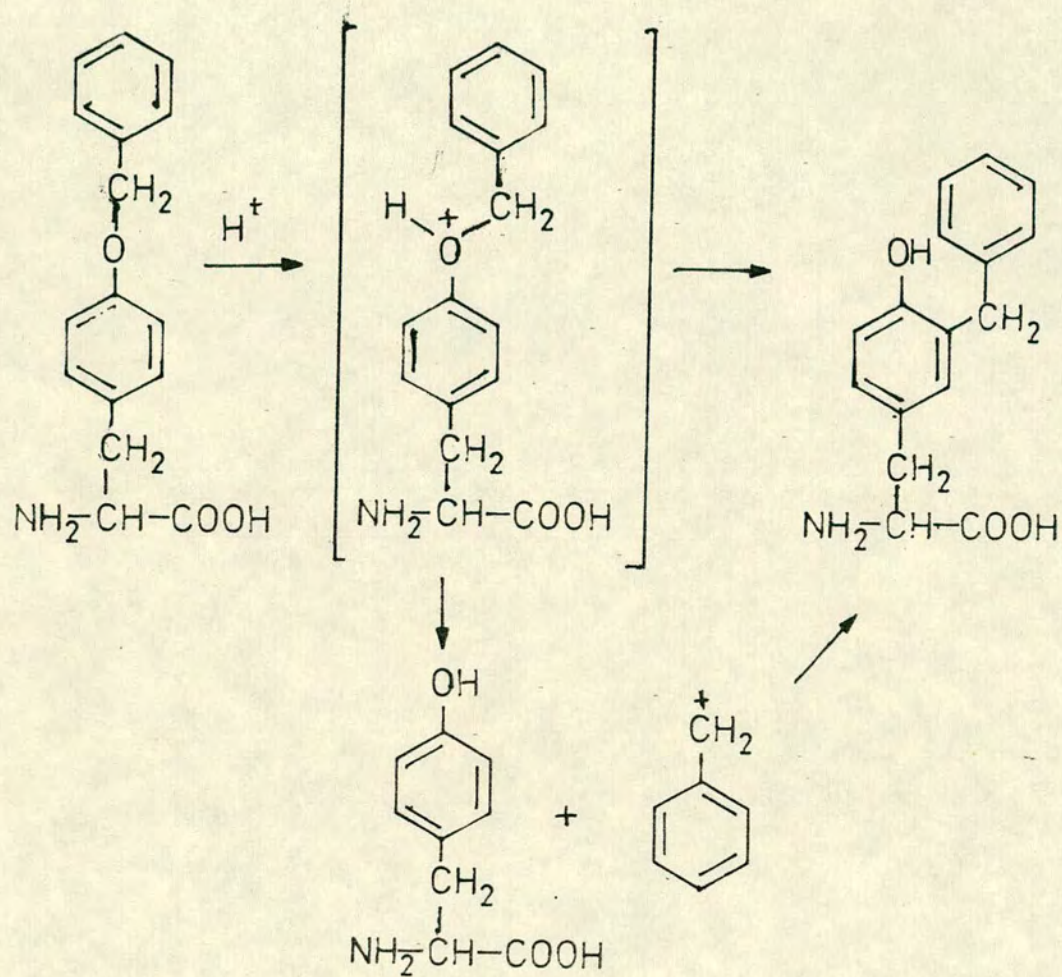
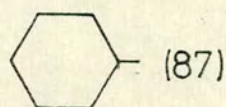
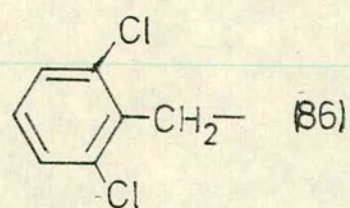
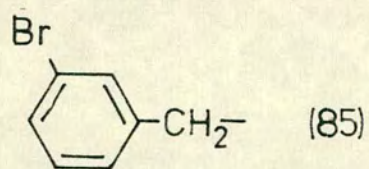


Fig.2.7



methionine sulfoxide containing peptides have been found in the crude product after work up as seen on thin layer chromatography (tlc) or high performance liquid chromatography (hplc).

In the past the tyrosine phenolic hydroxyl group has been protected by a benzyl function in conjunction with Boc protection on the α -amino group⁸⁶. Unfortunately the benzyl group is too labile in the presence of acids or nucleophiles and serious losses are incurred during Boc deprotection. HF at 0°C cleaves the benzyl group in 10 minutes but this was accompanied by serious side reactions⁸⁶. In the presence of trifluoroacetic acid (TFA) and HF rearrangements occur to form 3-benzyltyrosine byproducts (Fig.2.7). This reaction was originally thought to proceed *via* intermolecular and intramolecular mechanisms since the addition of scavengers such as anisole reduces the byproduct formation but does not totally eliminate it. However, recent studies have shown that the choice of solvent greatly affects the formation of 3-benzyltyrosine derivatives. Deprotections in TFA with scavengers can result in up to 10% byproduct formation whereas the same deprotection in acetic acid gives no rearranged byproducts but only the deblocked peptide. This suggests that the rearrangement occurs only by a intermolecular mechanism in which the solvent is an integral factor. To incur greater acid stability electron-withdrawing groups were introduced into the benzyl aromatic ring. The *m*-bromobenzyl group (85)⁸⁷ and the 2,6-dichlorobenzyl group (86)⁸⁸ are completely stable in standard Boc deprotection conditions and when treated with HF they form the 3-benzyl tyrosine derivative in yields of only 2-7% in the presence of scavengers. Recently protecting groups based on *sec*-alkyl ethers have been investigated as these were thought to form carbenium ions which would readily rearrange to a neutral nonalkylating product in preference to alkylating the phenyl ring of tyrosine. As a result the

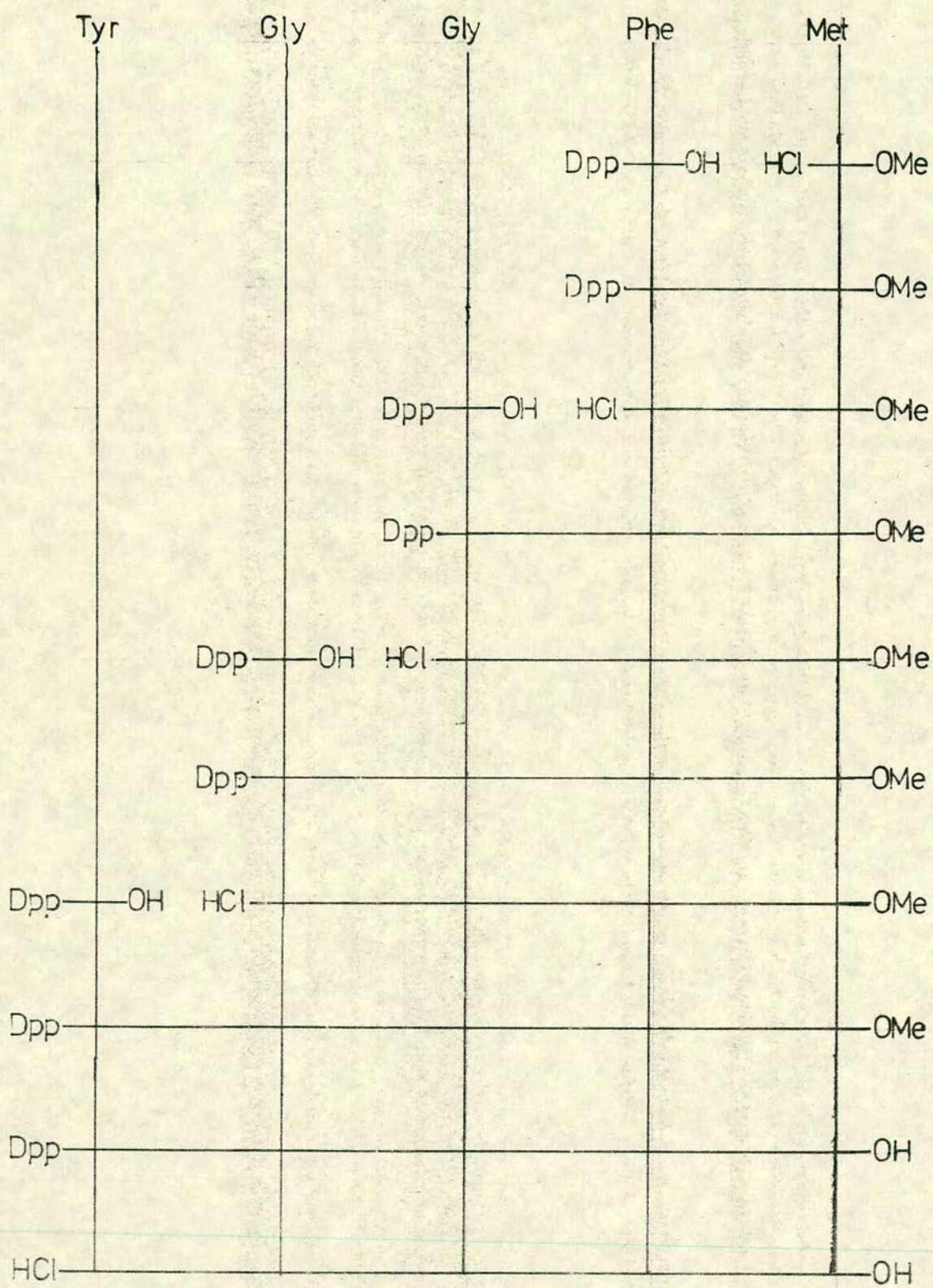


Fig.2.8

cyclohexyl group (87) has been shown to be suitably stable to Boc deprotection conditions, as well as nucleophiles commonly used in peptide synthesis, but it is easily cleaved using HF⁸⁹. Alkylation was found to occur when using neat HF but addition of scavengers reduced this to <0.5%. This group has been shown to be advantageous over the 2,6-dichlorobenzyl group in the synthesis of angiotensin II (88)⁸⁹ and fragments of histones H4 (89) and H3 (90)⁹⁰.

A novel way of protecting the phenolic hydroxyl group was the use of the *o*-nitrobenzyl group (91)⁹¹. The group is photolytically cleaved at a wavelength of 350nm in 10 to 12 hours at room temperature. This is indeed a very mild method of removing the protecting group. However accompanying the deprotection there was the formation of a yellow contaminant which could not be removed from the product, which might suggest why this group has not had widespread acceptance in the literature.

Due to the success of the previous synthesis of Met⁵-enkephalin⁶⁸ in which tyrosine has been used without any masking of the phenolic hydroxyl group and the proposal that this hydroxyl group does not undergo side reactions during mixed anhydride couplings⁹², it was decided to leave the hydroxyl function unprotected and to further explore the possibility of temporary protection during the final tyrosine coupling step.

2.6.4. Synthesis

Starting with MetOMe.HCl (92), Met⁵-enkephalin was made in a stepwise manner as shown in Fig.2.8.

Readily available MetOMe.HCl (92) was coupled to DppPheOH (93) using the general procedure outlined in "Carboxyl Activation" with the extra addition of 2,6-lutidine to yield Dpp-Phe-Met-OMe (94) (82%). Deprotection was carried

out at 35–40°C using a methanolic solution of hydrogen chloride and found to be complete within 15 minutes. A second deprotection using the same conditions at room temperature was also complete after 15 minutes. HCl-Phe-Met-OMe (95) could only be isolated as a brown oil and so was washed with anhydrous diethyl ether several times. The ether was decanted after each wash thus removing the diphenylphosphinic methyl ester byproduct (96). The oil was then dissolved in dry DMF and coupled immediately to DppGlyOH(97) in the same manner as before. Recrystallisation from chloroform hexane mixture afforded Dpp-Gly-Phe-Met-OMe (98) (70%) which was deprotected in the usual way to afford HCl-Gly-Phe-Met-OMe (99) (86%) as a white crystalline solid, after recrystallisation from methanol, diethyl ether. Coupling of HCl-Gly-Phe-Met-OMe to DppGlyOH yielded DppGly-Gly-Phe-Met-OMe (100) (60%) as a white solid which proved impossible to crystallise in a variety of solvent mixtures. Instead it came out of solution as a gelatinous precipitate. Tlc and hplc showed the compound to be pure and so was used without any further purification for the next coupling. Standard deprotection afforded HCl-Gly-Gly-Phe-Met-OMe (101) as an oil which was washed with diethyl ether and used immediately in the next step.

During the preparation of DppTyr(Dpp)OMe (102) following the literature procedure⁶⁸, it was noted that a white solid sometimes precipitated out during the aqueous work up. The solid was isolated and shown to be mainly (102) by spectral analysis and comparison with an authentic sample on tlc. This is not surprising since pure (102) is not very soluble in ethyl acetate. In a second preparation chloroform was used as the solvent in the organic phase and as a result the yield increased from 55%⁶⁸ to 85%. All further preparations of (102) used the modified work up procedure and consistently obtained higher yields than previously reported. Following the original method DppTyrOH.DCHA (103)

was prepared without incident (75%). Upon liberation of the free acid it was found that crystalline DppTyrOH (104) could be obtained by concentrating a dried ethyl acetate solution of it, acquired during the acidification of the salt, and cooling. Full spectral and elemental analysis showed the product to be DppTyrOH.2H₂O (105) (90%). This was troublesome when considering subsequent mixed anhydride activation since dry conditions are essential for the activation.

For the final coupling (105) was dissolved in dry DMF and dried 4A molecular sieves were added in an attempt to remove the water of crystallisation. Drying time was varied from half an hour to overnight but no significant changes in yield were observed. Also in this coupling two equivalents of diphenylphosphinyl chloride(DppCl) (106) was used to temporarily block the tyrosine phenolic hydroxyl group. Since previous attempts to isolate the bis-Dpp peptide directly from the reaction mixture resulted in obtaining a mixture of bis- and mono Dpp compounds (107,108) together with diphenylphosphinic acid (DppOH) (109) and methyl ester(DppOMe) (96), the usual work up involving acid and base washes was employed to afford solely the mono (α -amino) Dpp-peptide. Interestingly a coupling using one equivalent of DppCl gave the same mono Dpp peptide (108) in a comparable yield to the previous coupling. This would suggest that the phenolic group does not take part in any side reactions under the coupling conditions of the carboxylic phosphorus mixed anhydride as has been proposed in carbon based mixed anhydride couplings⁹². To study this further the reaction of DppTyrOH (105) with HClAlaOMe (110) using two equivalents of DppCl was followed by ³¹P nmr in order to examine the reaction profile. Because none of the intermediates can be isolated, following the reaction by ³¹P nmr does not give conclusive proof of the mechanism but can only support postulated mechanisms. HClAlaOMe was

chosen in preference to HClGlyOMe (111) due its greater solubility in DMF. Appendix I shows the results of this experiment. The anhydride formation is predictably instantaneous as seen by the appearance of a signal at 28ppm, however there is another signal very close to it . In some reactions DppCl was present ten minutes after its addition and the absence of a signal around 30ppm (which corresponds to the phosphorus of DppOPh (112) and the side chain Dpp phosphorus of DppTyr(Dpp)OMe (102)⁹ would suggest that the phenolic hydroxyl group is not blocked. The signal close to the phosphorus anhydride signal could be attributable to the Dpp-symmetrical anhydride (113) formed by the reaction of DppCl and DppOH.(The DppOH could arise from the hydrolysis of DppCl by trace amounts of water derived from either the solvent or DppTyrOH, due to incomplete drying. If all the water is used up excess DppCl will remain). Addition of the amine component in DMF resulted in a disappearance of the DppCl signal and the merging and enhancement of the signal at 28ppm. The DMF had been distilled and dried but might have contained a significant amount of water after being stored over a period of a few weeks before needing to be replenished. This would lead to partial hydrolysis of the DppCl to give DppOH which would react with the remainder of the DppCl to form the Dpp-symmetrical anhydride. No new phosphinamide signal at around 21-22ppm was observed which indicates the excess DppCl did not react with the liberated amine component of HClAlaOMe. This is not surprising since the formation of Dpp-phosphinamides takes two hours to be complete. Also, when comparing the strengths of the two new bonds formed in the phosphinamide and phosphinic acid preparations the P-O bond of the phosphinic acid is stronger than the P-N bond of the phosphinamide . Hence the phosphinic acid would be the predicted product. A signal at 16ppm appears soon after the addition of the amine component and can be attributed to the

diphenylphosphinate anion (DppO^-). As the reaction proceeds this signal increases with the concomitant decrease of the signal at 28ppm. This indicates the mixed anhydride is being consumed in the acylation reaction, the symmetrical anhydride remaining unchanged. Acylation of carboxylic-phosphorus mixed anhydrides are usually complete within two hours. However in this reaction there remained a signal at 28ppm, several hours after the addition of AlaOMe, which slowly decreased over 18 hours. This could be explained as the slow hydrolysis of the Dpp-symmetrical anhydride by water from the air or solvent. The experiment was worked up as usual and yielded a white powder, pure by tlc, which was shown to be Dpp-Tyr-Ala-OMe (114) by proton nmr.

The results of this experiment, plus the fact that coupling of DppTyrOH.2H₂O (105) to HCl-Gly-Gly-Phe-Met-OMe (101) proceeds with similar yields using either one or two equivalents of DppCl, tends to suggest that the phenolic hydroxyl group does not readily react with DppCl under the conditions of coupling and can be left unmasked. This is in contrast to previous work⁶⁸ done on Met⁵-enkephalin synthesis using Dpp-mixed anhydrides where (107) is reported to have been isolated. Clearly a more detailed study is required to fully understand what is happening.

After these preliminary studies (108) was made from DppTyrOH.2H₂O and HCl-Gly-Gly-Phe-Met-OMe using one equivalent of DppCl. After the usual work up the product was obtained by concentrating the dried ethyl acetate solution and cooling, resulting in a white precipitate which was pure by tlc and hplc (61%).

When comparing this synthesis of the fully protected Met⁵-enkephalin to the previous synthesis by D.Hopton⁶⁸, in which an extra equivalent of

TyrGlyGlyPheMet

Sephadex G 15
↓
5% AcOH_(aq)

TyrGlyGlyPheMet

Partition Chromatography Semi-Preparative
Sephadex G25 or RP18 hplc
↓

Tyr GlyGlyPheMet

purification of Met enkephalin

2,6-lutidine was not used, this synthesis has produced intermediate fragments, and a final product, of a much higher purity. This is exemplified in the final coupling by the synthesis of Dpp-Tyr-Gly-Gly-Phe-Met-OMe (108) which was originally obtained as a yellow powder after precipitation from a methanol solution by the addition of water whereas now it can be isolated as a white powder after crystallisation from ethyl acetate. In this case the yields of the coupling steps are not significantly greater than those reported in the previous synthesis.

Removal of the methyl ester function was achieved using 2.1 equivalents of NaOH in a DMF water,mixture (2:1) in six hours as shown by tlc to yield DppTyr-Gly-Gly-Phe-Met-OH (115) (91%). The Dpp group was removed using twelve equivalents of a 2M solution of hydrogen chloride in peroxide free dioxane,water mixture (2:1) at room temperature. The cleavage was followed by ^{31}P nmr spectroscopy and was shown to be complete after six hours. HCl.Tyr-Gly-Gly-Phe-MetOH (116) was purified by gel filtration on Sephadex G15 eluted with 5% aqueous acetic acid followed either by semi-preparative reverse phase hplc or partition chromatography on Sephadex G25 using butanol, acetic acid, water mixture (4:1:5) as the eluent. The slow cleavage is probably due to the solvent effects and the temperature of the reaction. Further work involving different dioxane,water mixtures or a different solvent such as 2,2,2-trifluoroethanol, together with varying the concentration of hydrogen chloride might prove very useful.

All nmr data of the larger peptides was obtained on a Bruker WH360 machine. Decoupling experiments were used successfully to assign all the signals. However, it was thought that this could be an ideal opportunity to evaluate the use of 2D COSY nmr and as a result 2D spectra of (115) and (116)

were obtained (Appendix II). They both show quite clearly all the relevant couplings, simplifying enormously the high field region (2–3ppm). Unfortunately the 2D spectrum of (116) did not show the α -CH signal of the tyrosine residue. This observation was attributed to interaction with the neighbouring NH_3^+ group.

CHAPTER 3

SUBSTANCE P

3.1. Isolation and Synthesis

Substance P (SP) (117) is an undecapeptide found throughout the central nervous system (CNS) and in peripheral organs. Over fifty years ago von Euler and Gaddum isolated a biologically active principle from the brain and gut⁹³. This extract stimulated the motility of the isolated rabbit ileum, even in the presence of atropine, demonstrating that the effect was not due to acetylcholine. These early studies were carried out using crude acid alcohol extracts of the equine brain and intestine, dried to a powder form. The active principle became known as "P" (for powder) and later universally accepted as "Substance P". Since it was first discovered much work has been done on the pharmacological properties of SP but it was not until forty years later that it was obtained in a pure enough form to be fully characterised and its structure determined, proving it to be a peptide. In the course of an attempt to isolate a corticotropin-releasing factor from the bovine hypothalami, Leeman and Hammerschlag⁹⁴ found a peptide showing sialogogic activity. Several of its properties were similar to SP and it was suggested that the sialogen might be SP⁹⁵. The emphasis of the work was changed to the isolation of the peptide and by the use of gel filtration, ion exchange chromatography and high voltage paper electrophoresis they were able to obtain pure samples. (The purity was checked by high voltage electrophoresis⁹⁶). Amino acid analysis showed it to be an undecapeptide composed of Lys₁ Arg₁ Gln₂ Pro₂ Gly₁ Leu₁ Met₁ and Phe₂ with the arginine as the amino terminal residue. All pharmacological properties of the peptide were identical to those of SP. The extract was of sufficient purity that they were soon able to sequence SP⁹⁷ by chymotryptic cleavage, Edman degradation and carboxypeptidase treatment, and reported its structure to be

Substance P	(117)	ArgProLysProGlnGlnPhePheGlyLeuMetNH ₂
Physalaemin	(118)	GlpAlaAspProAsnLysPheTyrGlyLeuMetNH ₂
Uperolein	(119)	GlpProAspProAsnAlaPheTyrGlyLeuMetNH ₂
Phyllomedusin	(120)	GlpAsnProAsnArgPheIleGlyLeuMetNH ₂
Eledoisin	(121)	GlpProSerLysAspAlaPheIleGlyLeuMetNH ₂
Kassinin	(122)	AspValProLysSerAspGlnPheValGlyLeuMetNH ₂

Fig.3.1

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂. Two years later Studer *et al*⁹⁸ isolated SP from horse intestine and purified it using established procedures^{96,99,100}. They found the amino acid composition and sequence of SP to be identical to the sialogogic peptide hence finally proving von Euler's SP and the sialogogic peptide to be the same compound. Chang and Leeman were also involved in the first synthesis of SP¹⁰¹, carried out using solid phase methodology¹⁰². Since then there has been several syntheses *via* both solid phase techniques^{102,103} and classical solution methods¹⁰⁴⁻¹⁰⁶. In each case the pure product was found to have identical properties to SP extracted from natural sources.

3.2. The Tachykinins

SP is in the tachykinin family whose members are found in many vertebrates and invertebrates (Fig.3.1). Analysis of the members of this family¹⁰⁷⁻¹⁰⁹ has shown them to have similar structures and biological activities. Common to these peptides is the C-terminal sequence Phe-Xxx-Gly-Leu-Met-NH₂ which seems to play an important role in their activity. In smooth muscle and the secretory organs they all have similar activities but the relative potencies do vary considerably. It is not yet fully understood how SP or the other tachykinins interact at the receptor sites but it has been suggested that there are at least two different types of receptors^{110,111}. This postulation is based upon the differing potencies of the tachykinins relative to SP. On one type of receptor, as found in the GPI, four tachykinins (physalaemin, SP, eledoisin and kassinin) were found equipotent. This type of receptor was called the SP-P receptor because physalaemin was usually the most potent, although all four peptides acted at nanomolar quantities. The other type of receptor, typified by the rat vas deferens, had a rank order of kassinin > eledoisin >> SP=physalaemin and was called the SP-E

receptor. However the major difference here was that while kassinin and eledoisin acted at nanomolar quantities, SP and physalaemin were used in concentrations of micromolar quantities in order to elicit a response. To assume there are two subclasses of receptors purely on the basis of the above findings is a little naive¹¹², since accessibility to receptors or different rates of inactivation could lead to differences in the relative potencies of tachykinins. However, in an attempt to make an analogue which would act specifically on one of the sites, and so add more weight to this postulation, Sandberg and his coworkers found that if the methionine carboxamide of SP was changed to the corresponding methyl ester the resulting analogue was equipotent with SP in SP-P systems but there was a large decrease in activity on bioassays of the SP-E type¹¹³. As to the presence of these subclasses in the CNS, the picture is not so clear. It has been reported that eledoisin is more potent than SP in eliciting scratching behaviour in mice¹¹⁴ and antidipsogenic activity in rats¹¹⁵, suggesting the presence of SP-E sites. However, the specificity of [³H]SP binding sites in the rat brain membranes seem to reflect that of SP-P sites but SP-E sites would only be expected to have a low affinity for [³H]SP and would not be detected in binding experiments. There is a good possibility that both subclasses are in the CNS but more work has to be done to make the picture clearer.

3.3. Biosynthesis and Substance K

Until recently SP was the only tachykinin in mammalian systems but the fact that other neuropeptides are known to be present in the peripheral and central nervous system and that non-mammalian kassinin and eledoisin are more potent than SP in some peripheral actions and central nervous effects tends to suggest other, hitherto unknown, tachykinins might be present in the nervous system. If this is so it is reasonable to assume that they would

...ArgArgProLysProGlnGlnPhePheGlyLeuMetGlyLysArg...

SP sequence

...LysArgHisLysThrAspSerPheValGlyLeuMetGlyLsArg...

SK sequence

Partial sequences of (123) and (124) respectively.

Substance K (125) HisLysThrAspSerPheValGlyLeuMetNH₂

Neurokinin α (126) HisLysThrAspSerPheValGlyLeuMetNH₂

Neurokinin β (127) AspMetHisAspPhePheValGlyLeuMetNH₂

originate from the same or similar precursors. It is generally accepted that SP is initially synthesised as a large precursor but very little is known about SP origin and relationship to other tachykinins. By cloning techniques and DNA-sequencing Nawa and his coworkers¹¹⁶ determined two types of bovine brain precursor. One, α -preprotachykinin (123), was found to have a SP sequence within it flanked by two basic amino acids. The other, β -preprotachykinin (124), also had the SP sequence flanked with two basic amino acids but also had a sequence which was very similar to kassinin. This sequence was flanked by two basic amino acids and so could be liberated from the precursor at the same time as SP. This sequence was called Substance K (SK) (125) and it was predicted to be the mammalian equivalent of kassinin or eledoisin type of tachykinins. SK is probably a decapeptide since a survey of various prohormones suggests that the Lys-Arg pair flanking the peptide sequence is generally removed during processing¹¹⁷, unlike the Arg-Arg pair preceding the SP sequence. Later work by Kumura and his coworkers¹¹⁸ led to the isolation of two neuropeptides called neurokinin- α (126) and neurokinin- β (127), from the porcine spinal cord. Neurokinin- α is a decapeptide with the same amino acid sequence as SK. Neurokinin- β is a very similar neuropeptide. These new tachykinins have been synthesised by Folkers *et al*¹¹⁹ using solid phase methodology. Neurokinin- α was found to have 81% of the activity of SP and neurokinin- β was found to have 65% of the activity of SP but this was only based on the GPI assay which is typical of SP-P type receptors. A more extensive study has since been carried out by Nawa and his colleagues¹²⁰. After recognising the SK sequence in the bovine brain precursor they synthesised SK and two analogues, and tested their biological activity. The data presented showed that SK had biological activity peculiar to the tachykinins and similar to kassinin. This could be accounted for by the similar C-terminal

sequences of the two peptides. When tested on several SP-P type and SP-E type receptors SK was more active than SP on the latter type and less active on the former. Thus they concluded that SK represents a second type of mammalian tachykinin that serves as an endogenous ligand for the SP-E receptor and may be mutually related but have different physiological roles in the mammalian environment.

3.4. Distribution and Pharmacological Properties

SP has been found in the central and peripheral nervous system of many mammalian species^{121,122}. In the CNS it has been detected in over thirty parts of the brain, the spinal cord and many parts of the brain stem. Large concentrations have been found in the mesencephalon hypothalamus¹²²⁻¹²⁷.

In the peripheral nervous system SP has been found in many varying areas. Peripheral tissues such as the skin¹²⁸, lip¹²⁹, tongue¹³⁰ and tooth pulp¹³¹ of various mammals have all been found to have fibres containing SP. Optic nerves and cells in the retina, iris, stroma and the optic nerve itself contain SP^{132,133}. Many studies, mainly on the rat and guinea pig, have found SP containing fibres and cells in varying amounts throughout the gastrointestinal tract from the oesophagus, stomach, duodenum, jejunum and ileum to the colon and rectum¹³⁴.

Since SP is found in many different areas within the body it is not surprising that it has numerous pharmacological effects usually dependant on location and dose. One of the properties first observed when it was originally isolated was its stimulating effect on most smooth muscle layers in the gastrointestinal tract. It has been found to contract all segments of the small intestine in many mammalian and cold blooded species. The relative effect of SP is paralleled with its natural abundance within these segments. There is a

dose dependant contractile effect in circular smooth muscle of the cat oesophagus, and the fundus and corpus of the guinea pig stomach, but not in the longitudinal muscle of the fundus and corpus. Interestingly, there is also a greater density of SP positive fibres in circular smooth muscle than in longitudinal muscle of the rat stomach^{135,136}. In contrast the isolated circular muscle of the guinea pig ileum does not evoke any contractions at low concentrations¹³⁷ whereas the isolated longitudinal muscle of the guinea pig ileum evokes a tonic contraction with doses as small as 0.7pmol¹³⁸. From the gastrointestinal sphincter to the ileum there is an increase in its maximal effect as there is also a similar rise in SP concentration in these segments¹³⁹.

SP stimulates secretion in the salivary glands and the pancreas¹⁴⁰. *In vivo* and *in vitro* experiments on the dog, rat, guinea pig and hamster have shown an increase in salivary flow from the mouth and nose when SP is injected intravenously^{94,141}. The dose dependant effect is a direct action on the salivary glands as it is not blocked by atropine (128), propranolol (129) and phenoxybenzamide (130) which would prevent any response due to acetylcholine (131) or histamine (132) released by SP. From *in vivo* and *in vitro* experiments in the dog SP has shown to stimulate secretion of pancreatic juices in the exocrine pancreas by as much as 160%¹⁴². Little is known how SP is involved in this effect from the limited information available. The presence of SP in the central and peripheral nervous system led several workers to think that SP was involved in the transmission of pain. Early work showed that introduction of SP onto neurons in the spinal cord caused an increase in the firing rate of cells that are also excited by noxious stimuli, which tended to suggest that SP was directly involved¹⁴³. More recent studies seem to show that the involvement of SP in pain transmission varies with its location in the nervous system. In the sympathetic ganglia SP has been shown to be directly

involved as it is released from synapses in the prevertebral ganglia and generates noncholinergic, slow excitatory postsynaptic potentials¹⁴⁴. Similar effects have been shown to occur when SP has been found in neurons within the spinal cord¹⁴⁵. However, other evidence has tended to suggest SP might have a role as a neuromodulator. Removal of up to 60% of SP from the trigeminal nucleus caudalis by neonatal treatment with capsaicin in the rat did not alter the proportion of neurons responding to noxious and non-noxious stimuli¹⁴⁶. This would suggest that SP is not the only compound responsible for pain transmission. Other peptides such as somatostatin and vasoactive intestinal polypeptide have been found in small sensory neurons and until their exact role is known SP cannot be assigned as the sole transmitter of nociceptive information.

The possibility of SP being an analgesic was first proposed by Stewart¹⁴⁷ who reported long lasting analgesia in mice (using the hot plate test) after intracerebroventricular and intraperitoneal SP injections. This effect which was prevented by naloxone was confirmed by later work with mice and rats^{148,149}. However, some other authors found no analgesia, if anything a decrease in reaction time to the hot plate test, under apparently the same conditions^{150,151}. These contradicting results of SP are apparently dose dependant. Small doses of SP (<4pmol administered to the lateral ventricle) produce naloxone dependant analgesia probably by releasing endogenous opioid peptides. Larger doses (>40pmol) give rise to hyperalgesia effects by stimulating neuronal activity in nociceptive pathways.

Since positive fibres and cells are usually associated with blood vessels all over the body it is not surprising that injections of SP can change the dynamic equilibrium of the blood network. Work done to date on the dog has shown SP

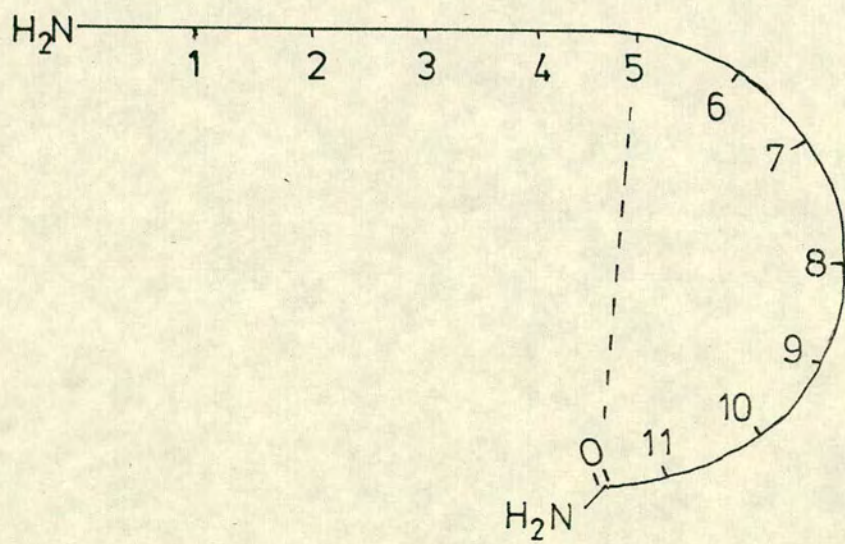


Fig.3.2

to be a very potent vasodilating compound. Close arterial injections causes vasodilation in the adipose tissue and skeletal muscle¹⁵². Intravenous injections in dog increases the blood flow in most vascular beds such as the skin, skeletal muscle and small intestine¹⁵³. Intravenous injections in man causes a similar increase in blood flow with red flushing of the skin around the face and neck¹⁵⁴. in circular smooth muscle than longitudinal muscle of the rat stomach^{135,136}.

3.5. Substance P Analogues

3.5.1. Structure Activity Studies

From early nmr evidence SP and biologically active C-terminal fragments were found to contain a hydrogen bond between one of the Gln residues and a carboxyl in the C-terminal region and also one of the phenylalanines has a constrained conformation¹⁵⁵. These properties were not found to be present in shorter inactive fragments. Thus it was concluded that SP had a U-shaped peptide backbone in which probably the Phe⁷ side chain had the constrained orientation (Fig.3.2). This has since been backed up by conformational energy calculations¹⁵⁶ which suggested that favoured bioactive structures are stabilised by interactions between the C-terminal residue and the Gln⁵ or the Gln⁶ residues, and by molecular structures of SP based on a semi-empirical conformational analysis¹⁵⁷ which suggests a bend in the peptide backbone near Phe⁷ and Leu¹⁰. Recent work involving nmr and circular dichroism (cd) studies suggests that in water SP has many complex conformations as well as aggregation with itself¹⁵⁸. However in methanol SP adopts one major conformation where the Arg-Pro-Lys portion is very flexible, the Pro-Gln-Gln-Phe-Phe portion is part of a α -helix and the Gly-Leu-Met is orientated so that there is some interaction with the Gln^{5,6} primary amides.

Taking all these reports into account the overall picture is that SP and active fragments have a U-shaped peptide backbone with a conformationally constrained Phe⁷ which could be essential for fitting and binding to receptor sites. Whether this is the actual conformation of SP at the receptor site or merely a solvent induced conformation cannot be determined from the above work.

This proposed bioactive conformation of SP would explain why shorter C-terminal fragments have comparable biological activity which would seem to question the need for the N-terminal tetrapeptide¹⁵⁹. However there are several properties of SP which have been attributable to this area of the peptide. Substitution of D-amino acids in the C-terminal region of C-terminal fragments removes the hydrogen bonding which results in loss of biological activity¹⁶⁰. D-amino acid substitution in the C-terminal region of SP results in analogues with no hydrogen bond but with a significant amount of biological activity. This would suggest that the N-terminal tetrapeptide stabilises the bioactive conformation. Other studies have shown that the N-terminal tetrapeptide also stabilises SP against enzymic degradation¹⁶¹. C-terminal hexa- and hepta-peptides were shown to have comparable activities to SP but in the presence of rat brain homogenates, which contain peptidases, had a much shorter lifetime. Finally SP induced histamine release has been attributed to the N-terminal tetrapeptide¹⁶². Whilst C-terminal fragments are devoid of histamine-releasing activity, the N-terminal tetrapeptide did cause histamine release and the effect was increased when it was coupled to various C-terminal fragments¹⁶³.

From the little work done on studying positions 5 and 6 it can be seen that the amide functionalities of both Gln residues do not participate in maintaining

the bioactive conformation. The biological activity of analogues with L-alanine substituted in either position 5 or 6 (134,135) was similar to SP on *in vivo* and *in vitro* preparations¹⁶⁴. C-terminal hexa- and heptapeptides with an N-terminal pyroglutamic acid gave increased and equal biological activity (135,136), respectively¹⁶⁵. An analogue with N,N-dimethylglutamine in position 6 (137) had equal biological activity to SP. All these analogues have little or no amide functionality on the side chain but all retain full biological activity. Phe⁷ is an interesting position in that it occurs in all tachykinins and is thought to be directly involved with binding to the receptor. From the work that has been done replacement of the Phe with a neutral aliphatic amino acid (Leu or Ile) drastically reduces the biological activity¹⁶⁶. Changing the nature of the aromaticity of the phenyl ring by introducing electronegative and electron-donating substituents in the *para*-position did not change the biological activity¹⁶⁷. Substitution of Phe with cyclohexylalanine (CHA) resulted in only a small drop in biological activity. These results suggest the aromatic character and planar structure of the phenyl ring are not essential for high biological activity¹⁶⁶. The phenyl side chain in position 8 has been found not essential for biological activity. Substitution with any neutral amino acid does not result in a loss of activity¹⁶⁸. Interestingly, SP (117) and physalaemin (118) have aromatic residues in this position and eledoisin and kassinin have aliphatic residues in this position, hence analogues with a selectivity for SP-P or SP-E receptors could be envisaged with appropriate substitutions in this position. However, this was found not to be the case when [Ala⁸]SP¹⁻¹¹ (138) was tested on the GPI assay (SP-P type receptors) or the rat vas deferens (SP-E type receptor). 30-40% of the activity of SP was observed on both assays¹⁶⁸. Gly⁹ is important due to the bend in the peptide backbone at this position. Analogues with N-methylglycine or L-alanine have equipotent

biological activity^{168,169} but D-amino acid substitution results in analogues with no biological activity¹⁷⁰. Hence, Gly⁹ is not important as a special conformational element (since L-amino acid substitutions do not destroy biological activity) but the glycine does not adopt a pseudo D conformation. Substitution of Leu¹⁰ by Ala results in a slight loss in biological activity¹⁶⁸ but unfortunately no other work has been published about this position. Originally substitution of Met¹¹ with most L-amino acids resulted in a loss of biological activity on the GPI assay. Further studies on changing the nature of the methionine side chain by introducing other hetero atoms (O,C,Se) and branching showed only one analogue, in which Se replaces the S, to have biological activity¹⁷¹. Thus it was suggested that the lipophilicity and van der Waal's radius at the δ position might be the active elements. Substitution of Leu for Met¹¹ has recently been shown to increase the antagonistic activity of known antagonists even though it has no agonist activity as it was originally discovered (see later for more detail). The C-terminal amide has been shown to be essential for activity. SP¹⁻¹¹COOH (139) is completely devoid of activity¹⁷² whereas SP¹⁻¹¹COOMe (140) shows equipotent activity with SP on SP-P type receptors but no activity on SP-E type receptors¹¹³. Also (140) has a greater activity than SP in the CNS¹⁷³.

3.5.2. Enzyme Resistant Analogues

Many analogues of SP have been found to have equal or greater biological activity but have a short lifetime in *in vivo* preparations. This is equally applicable to antagonists where analogues have been made with antagonistic activity, as seen in *in vitro* assays, but are useless in *in vivo* experiments due to rapid enzymic degradation¹³⁴. With this in mind work has been done to design enzyme resistant analogues. Purified extracts of endopeptidases from within the brain have been shown to cleave SP at the Gln⁶-Phe⁷, Phe⁷-Phe⁸

and Gly⁹-Leu¹⁰ amide bonds. A membrane-bound endopeptidase, thought to be involved in the removal of SP after synaptic release, has been isolated and shown to degrade SP but not other neuropeptides¹⁷⁴. In order to stop the amide bond cleavage, analogues with N-methyl amino acids in the relevant positions have been made. These analogues were resistant to cleavage at the aforementioned bonds¹⁶⁸ but the N-terminal fragment was cleaved¹⁷⁵. Therefore analogues based on C-terminal hepta- and hexa-peptides with a N-terminal pyroglutamic residue were tested. [Glp⁵, (Me)Phe⁸, (Me)Gly⁹] SP⁵⁻¹¹ (DiMe-C7) (141) was equipotent with SP in the rat brain but completely resistant to the SP-degrading membrane-bound peptidase^{169,175}. Similarly, [Glp⁶, (Me)Phe⁷, (Me)Leu¹⁰]SP⁶⁻¹¹ (142) had the same biological activity as SP but was resistant to degradation by pepsin, chymotrypsin, papain and thermolysin¹⁷⁶.

3.5.3. Antagonists

Work to date, on designing and synthesising SP antagonists has been based on modifying the peptide backbone by D-amino acid substitution. This proved very useful when key amino acids of luteinizing hormone releasing hormone (LHRH) were replaced by D-amino acids to yield a potent antagonist¹⁷⁷. Early work centred on structure activity studies using single D-amino acid substitutions in order to form an analogue with no agonist activity. As a result, D-amino acid substitutions in C-terminal fragments and SP in positions 2,7,8,9 and 11 gave promising results. [D-Phe⁷]SP¹⁻¹¹(143) and [D-Pro²]SP¹⁻¹¹(144) are full length analogues with no agonist activity¹⁷⁸. Using the results of the LHRH work D-Trp was substituted into C-terminal fragments but only one analogue, [Arg⁶, D-Trp¹⁰]SP¹⁻¹¹(142), had no agonist activity, as well as marked antagonist activity¹⁷⁹.

1	D-Arg	Pro	Lys	Pro	D-Phe	Gln	D-Trp	Phe	D-Trp	Leu	Leu	NH ₂
2	"	"	"	"	Gln	"	"	pCl Phe	"	"	"	"
3	"	"	"	"	D-Phe	"	"	"	"	"	"	"
4	"	"	"	"	Gln	"	"	Phe	"	"	N!e	"
5	"	"	"	"	D-Cl ₂ Phe	"	"	"	"	"	"	"
6	"	"	"	"	D-Nal	"	"	"	"	"	"	"
7	"	"	"	"	D-Phe	"	"	pCl Phe	"	"	"	"

Fig. 33

To design analogues with antagonist activity D-Trp was substituted into two or three key positions within SP. From the many full length analogues originally made [DPro²,D⁷Phe⁷,DTrp⁹]SP¹⁻¹¹(146) and [DPro²,DTrp^{7,9}]SP¹⁻¹¹(147) were found to have antagonistic activity with weak agonistic effects¹⁸⁰. Multiple substitution of DTrp in C-terminal fragments of varying lengths led to the development of several analogues with increased antagonistic activity. [DPro⁴,DTrp^{7,9}]SP⁴⁻¹¹(148), an analogue very similar to [DPro²,DTrp^{7,9}]SP¹⁻¹¹(147) but much more potent, was also found to have no agonistic activity¹⁸¹. [DPro⁴,DTrp^{7,9,10}]SP⁴⁻¹¹ (149) with DTrp substituted in all the key positions has 3-6 times the antagonistic activity of its predecessor without any agonist effects. It has been shown to be active against the stimulatory effects of SP (in the GPI) and the inhibitory effects of SP (the rat blood pressure, the rabbit isolated heart and the dog carotid artery¹⁸¹).

In search for an antagonist with no agonist activity it has been found that D-amino acid substituted analogues with Met¹¹ replaced by Leu show enhanced antagonistic activity with no agonist effects. This was first noted when [DArg²,DTrp^{7,9},Leu¹¹]SP¹⁻¹¹(150) was found to have increased antagonistic activity¹⁸², sometimes as much as a 100-fold, but no agonistic activity. Recent developments of this analogue, mainly by small changes in the aromaticity of the side chain of position 7, has led to twenty more similar analogues with increased antagonistic activity¹⁸³. (Fig.3.3 shows some of the more potent analogues). C-terminal fragments based on [DPro⁴,DTrp^{7,9}]SP⁴⁻¹¹ and [DPro⁴,DTrp^{7,9,10}]SP⁴⁻¹¹ with Met¹¹ replaced by Phe, Leu and Nle have also been made and shown to have greater antagonistic activity over their predecessors, without a trace of agonistic activity¹⁸⁴. All of them have been shown to be a more potent antagonist than [DArg², DTrp^{7,9},Leu¹¹]SP¹⁻¹¹ in various *in vitro* and *in vivo* assays.

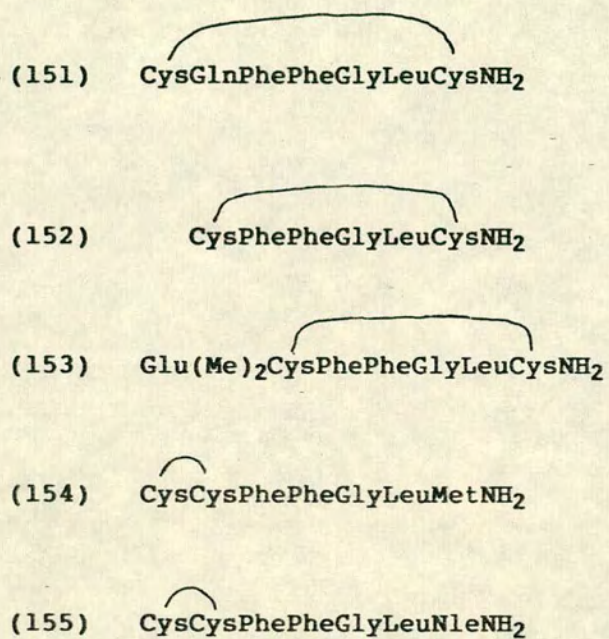
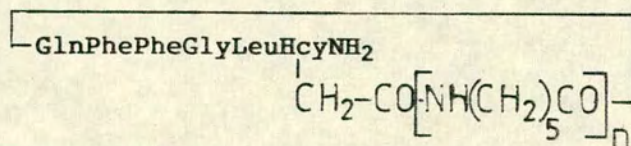


Fig.3.4



$$n=012$$

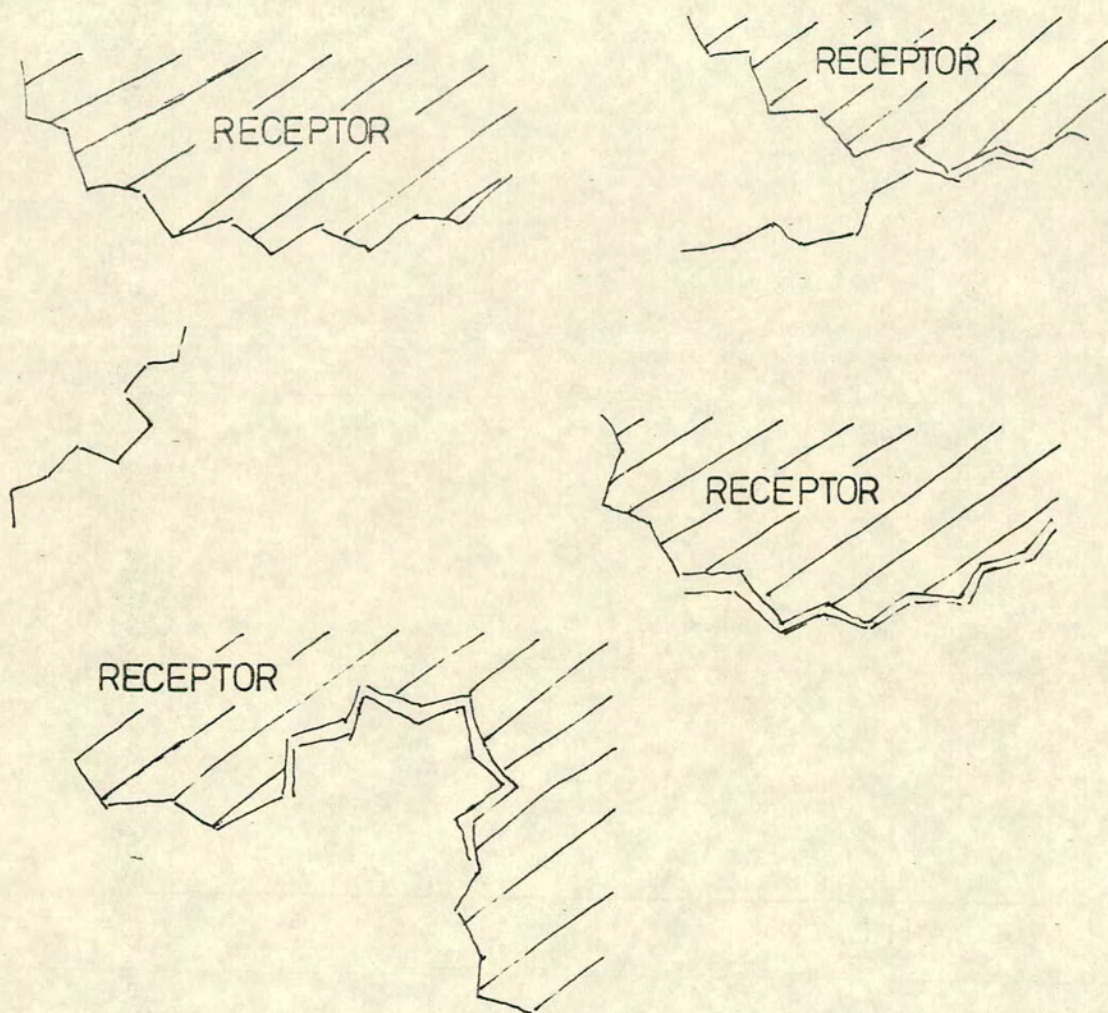
Fig.3.5

To date there are several antagonists available specific only to SP for biological testing. However, their antagonistic activity and potency are not of sufficient magnitude for them to be considered as models for drug design and so they can only be used as a basis of future work.

3.5.4. Cyclic Analogues of SP

Since the active conformation of SP was thought to be a U-shape with some interaction (ie H-bonding) between the C-terminal carboxamide and the Gln's in positions 5 and 6 cyclic analogues were made in an attempt to fix this shape, so as to conserve the bioactive conformation in different solvents and media, which would hopefully enhance agonistic activity.

To date there have been two approaches to the synthesis of such analogues. The first substitutes appropriate amino acids, where the connection is to be made from, with cysteine and then forms a disulphide bridge to make the link^{185,186}. as in the compounds listed in Fig.(3.4). Surprisingly no activity was found in compounds (151-153) and (154,155) only possessed a very small ^{amount} of agonist activity. Other methods of forming a link included an amide bond linkage between glutamic acid and lysine in the appropriate positions¹⁸⁷ and spacer arms made up of ϵ -amino caproic acid units¹⁸⁸. With the latter method the distance between positions 5 (or 6) and 11 could be varied (Fig.3.5). These analogues were also found to be weak agonists or to have no activity at all. Hence it seems that the SP receptors must recognise SP in an extended form and bind to it in a zipper like fashion, after which its conformation may change to the U-shape for binding to the active site. If this was so the linkage of positions 5 (or 6) to 11 is clearly too rigid and a more flexible connection is required.



Zipper Type Binding

3.6. Discussion

As part of a programme, aimed at the synthesis and evaluation of potential SP antagonists, being carried out within our research group, this work has involved the preparation of analogues which might disrupt the biosynthesis of the C-terminal amide. In conjunction with other work, C-terminal fragments of SP and SP-COOH were also synthesised. It was thought that biological activity might be as a result of favoured structural conformations which could be observed through interpretation of nmr data. In addition to this the synthesis and development of potential reagents for peptide synthesis (such as new phosphorus activating groups, a novel method for binding peptides to solid supports and the use of spacer arms in solid phase peptide synthesis), was carried out to varying degrees.

3.6.1. Peptide Synthesis

The synthesis of SP C-terminal fragments and extended fragments was seen as another opportunity to further demonstrate the use of the Dpp group as an acid labile amino protecting group and organophosphorus reagents as activating groups. Of the amino acids present within the C-terminal sequence of SP methionine is the only one which might be considered for side chain protection. With it being at the carboxy terminus it would be vulnerable to attack by any reactive intermediates formed during the repetitive deprotection of α -amino protecting groups. (This could be a problem if Boc was used as an α -amino protecting group). However, with the use of Dpp as the α -amino protecting group which does not form any reactive intermediates during deprotection, and the success of the Met⁵-enkephalin synthesis in which there is also a C-terminal methionine residue, it was decided not to mask the methionine side chain in any way.

Dpp- group

From previous work it had been shown that the Dpp group can be removed from a peptide with a C-terminal amide without any transesterification¹⁸⁹. Standard conditions for such a deprotection are six equivalents of hydrogen chloride in methanol or trifluoroethanol at 0°C for six to eighteen hours. These reactions have been monitored by tlc and ³¹P nmr spectroscopy. The long reaction times for complete cleavage led us to use the methyl ester for carboxyl protection which enabled us to use the standard deprotection conditions outlined in the previous chapter. Removal of the methyl ester was as before using one equivalent of NaOH to give the free acid and where necessary the methyl ester could be amidated using a saturated solution of ammonia in methanol to produce the C-terminal amide.

For the stepwise synthesis of some of the fragments the procedure was the same as that employed for the synthesis of Met⁵-enkephalin. The synthesis of two of the larger analogues involves a fragment condensation between a pentapeptide and a tetrapeptide. Fragment condensations allows the synthesis of large peptides to occur on a larger scale without the need to make vast amounts of smaller fragments and starting materials, as is required for stepwise synthesis. A problem associated with fragment condensations is racemization of the activated residue. This can occur in two ways, either by base abstraction of the α C-H proton or by formation of a 5(4*H*)-oxazolone. Activation of the carboxyl function is usually achieved by an increase in the thermodynamic activation of the acyl function which results in an increase in the lability of the α -proton. This proton is then prone to abstraction by any bases present. However, strong bases usually exist as salts and in the presence of the activated carboxyl group the nucleophilic amino component will always take part in the preferred aminolysis. As for the 5(4*H*)-oxazolone formation this reaction is dependent on many factors namely the solvent, activating group,

bases present and the nature of R' and R'' (Fig.3.6).

The applicability of the Dpp group and the Cpt group to fragment couplings has recently been explored. Initial results on the use of the Dpp group suggest that it is too active, rendering the α C-H very labile as well as promoting formation of the 5(4*H*)-oxazolone. Experiments have shown racemization to occur from 6% to as much as 42%^{68,190}. A more detailed study on the use of the Cpt group in fragment couplings has shown that when used with N-ethylmorpholine (156) as the base in either methylene chloride or DMF only 1.5% racemization occurs¹⁹¹. The proposed fragment condensation involved the activating of a glycine residue on the N-terminal fragment, which overcomes the problem of racemization of the activated residue. Formation of oxazolones results in lower yields and the unused liberated amines can be involved in minor side reactions lowering the yield still further and causing difficulty in isolation of the pure product. Therefore CptCl (157) was used under the conditions proposed by Ashton for fragment coupling condensations¹⁹⁰.

3.6.2. SP Extended Analogues

Since it is known that amidating enzymes exist which oxidatively convert C-terminal glycines to an amide functionality when processing peptides from their precursors (Fig.1.3), and such a process has been postulated for SP, it was considered that the synthesis of extended SP analogues with residues which would protect the Met¹¹-Gly¹² bond from amidation might provide useful antagonists. As the C-terminal heptapeptide fragment of SP has been shown to have comparable activity to SP itself, but does not have the histamine releasing properties of the N-terminal tetrapeptide, all the analogues are based on this sequence. Originally, before the mechanism of amidation was fully understood, it had been observed that extending the C-terminal peptide amide by two

DppPheGlyLeuMetGlyGlyOMe/OH	(169)/(170)
DppGlyLeuMetGlyGlyOMe/OH	(166)/(167)
DppLeuMetGlyGlyOMe/OH	(163)/(164)
GlpGlnPhePheGlyLeuMetGlyGlyOMe	(172)
GlpGlnPhePheGlyLeuMetGlyGlyOMe	(173)

Fig.3.7

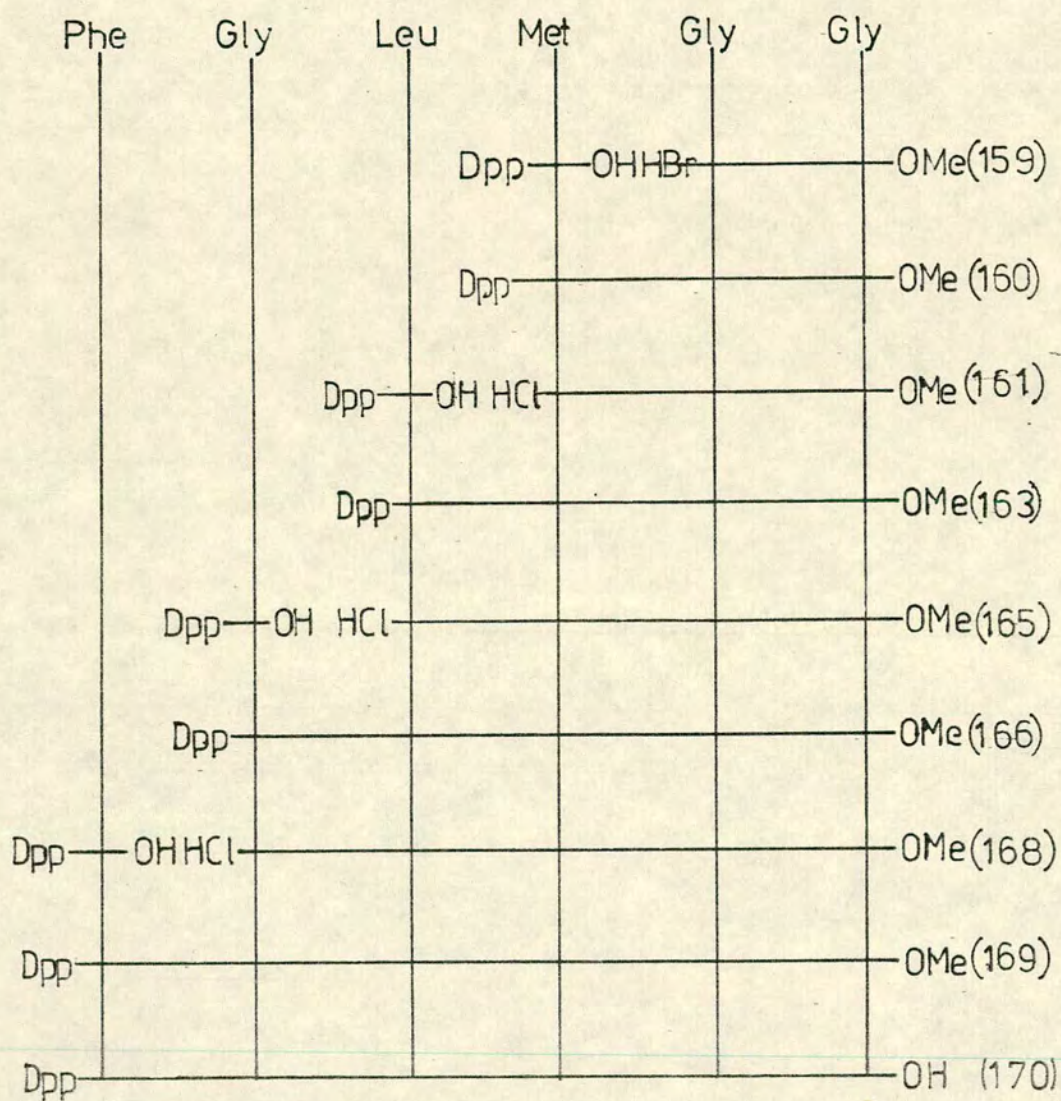


Fig.3.8

glycines had reduced amidation. So, applying this to the SP case led to the synthesis of the fragments in Fig.3.7 all possessing two extra glycines on the C-terminus. This would result in Gly¹³ being involved in an amidating step resulting in a glycine extended peptide amide whose biological activity in SP assays is not known, but it may be an antagonist to SP. Thus any drop in agonist activity or the presence of antagonist activity might be due to the interaction of the extended analogue with SP amidating enzyme, thus disrupting the SP biosynthetic pathway. Future work in this area involves the strengthening of the Met¹¹-Gly¹² linkage and irreversible binding of the analogue to the amidating enzyme. This is hoped to be accomplished by replacing the glycine-N for C and synthesising glycine analogues which mimic possible reactive intermediates which could bind irreversibly to the active site in the amidating enzyme.

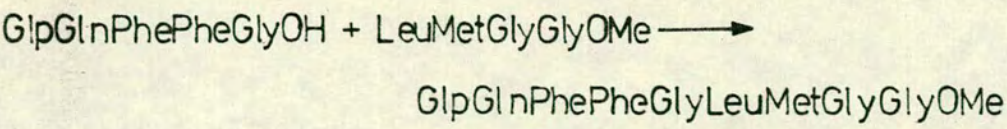
The smaller SP extended C-terminal fragments were made in a stepwise manner as shown in Fig.3.8. This work was started before the advantages of adding an extra equivalent of 2,6-lutidine were known. Otherwise the coupling and deprotection procedures were identical to those used for the synthesis of Met⁵-enkephalin. Thus DppMetOH (158) was coupled to HBr-Gly-Gly-OMe (159)¹⁹⁰ to yield crude DppMet-Gly-Gly-OMe (160) as a brown foam. Purification was achieved by dry flash chromatography using a stepwise gradient of 1% increments from 0-5% methanol in chloroform to give a white foam, yield 93%. Deprotection of the Dpp group gave a white powder after work up which was recrystallised from methanol-ether mixtures to give HCl.Met-Gly-Gly-OMe (161) in 85% yield. (161) was coupled to DppLeuOH (162) to give DppLeu-Met-Gly-GlyOMe (163) in a 70% yield after purification by gel filtration on Sephadex LH20 with methanol as the eluent or dry flash chromatography using the same elution conditions as before. (163) with the



two C-terminal residues of SP was thought big enough to be recognised by the amidating enzyme and so a portion of it was saponified to give DppLeu-Met-Gly-GlyOH (165). After workup (165) was obtained impure and was purified by wet flash silica chromatography (9:1,CHCl₃:MeOH) to give the pure product in a final yield of 84%. Attempts at recrystallisation gave only gelatinous precipitates. Deprotection of (163) took two hours at room temperature to yield HCl.Leu-Met-Gly-GlyOMe (165) as a white solid which was very hygroscopic. No further attempts were made to isolate and characterise this solid but instead after several washes with anhydrous diethyl ether and drying in a dessicator over phosphorus pentoxide overnight it was coupled in a DMF solution to DppGlyOH (97). Initially this was a troublesome coupling giving a crude product which was insoluble in ethyl acetate and so was applied to a Sephadex LH20 gel filtration column using DMF as the eluent. The product containing fractions were pooled to give a slightly impure product in about 60% yield, which after purification on silica resulted in a large loss in weight of material to give (166) in yields of round 40%. Changing the solvent from dichloromethane (DCM), DMF mixtures to DMF only, did not seem to make any difference on yield or purity. When this preparation was later repeated using an extra equivalent of 2,6-lutidine with DMF as the sole solvent the final yield was increased to 55% but no improvement on this could be made. Surprisingly saponification of (166) using the same conditions as for (164) took thirty minutes to give DppGly-Leu-Met-Gly-GlyOH (167) which was purified on a Sephadex LH20 column with DMF as the eluent in a final yield of 61%. Dpp deprotection of (166) proceeded smoothly in thirty minutes at 35-40°C affording a white hygroscopic powder of (169) which was stored *in vacuo* over phosphorus pentoxide until needed. HCl.Gly-Leu-Met-Gly-GlyOMe (168) was coupled to DppPheOH(93) in the usual manner without any incident affording a

crude product which was soluble in ethyl acetate and worked up as usual. DppPhe-Gly-Leu-Met-Gly-GlyOMe (169) was obtained as a white powder after precipitation from ethyl acetate by light petroleum ether in a 73% yield. Saponification of (169) to DppPhe-Gly-Leu-Met-Gly-GlyOH (170) was complete after four hours. The product was obtained as a white powder in a 68% yield.

From other work being done on structure activity studies of SP C-terminal fragments Glp-Gln-Phe-Phe-GlyOH (171) was available in relatively large amounts. Therefore the synthesis of Glp-Gln-Phe-Phe-Gly-Leu-Met-Gly-GlyOMe (172) and Glp-Gln-Phe-Phe-Gly-Leu-Leu-Gly-GlyOMe (173) was adapted to include a fragment condensation. The overall scheme is shown in Fig.3.9. Leucine was incorporated into position 11 after noting the reported enhanced antagonistic activities of analogues resulting from the work of Folkers¹⁵⁰ and Regoli¹⁸⁴. DppLeu-Met-Gly-GlyOMe (163) was prepared as before without incident. DppLeuOH was coupled to HBrGly-GlyOMe to yield DppLeu-Gly-GlyOMe (174) as an off-white foam after the usual aqueous work up. This was immediately deprotected using the standard procedure to give HClLeu-Gly-GlyOMe (175) in a yield of 64% after recrystallisation from methanol. Coupling of (175) to DppLeuOH (162) gave DppLeu-Leu-Gly-GlyOMe (176) as a white powder which was slightly impure after work up but could be purified by gel filtration on a Sephadex LH20 column eluted with methanol or by dry flash silica chromatography using the elution conditions as for (160). Either way produced the product in a yield of 73-80%. Attempts at isolating the hydrochloride salt of the deblocked compound (177) gave a white hygroscopic powder which proved troublesome to handle. Therefore HClLeu-Leu-Gly-GlyOMe (177) was prepared prior to the coupling step. Initial fragment couplings of (171) to (165) or (177) gave poor yields of the final product. In a typical preparation (171) was dissolved in DMF and cooled to



Lêu	Leu	Gly	Gly	
	Dpp—OH	HBr—	—OMe	
	Dpp—		—OMe	(174)
Dpp—OH	HCl—		—OMe	(175)
Dpp—			—OMe	(176)
HCl—			—OMe	(177)

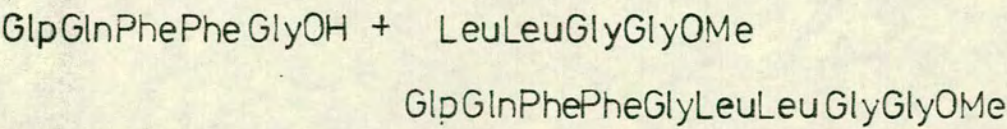


Fig.3.9

DppPhePheGlyLeuMetOMe/NH₂ (188)/(189)
 DppPheGlyLeuMetOMe/OH/NH₂ (184)/(185)/(186)
 DppGlyLeuMetOMe/OH/NH₂ (180)/(181)/(182)

Fig.3.10

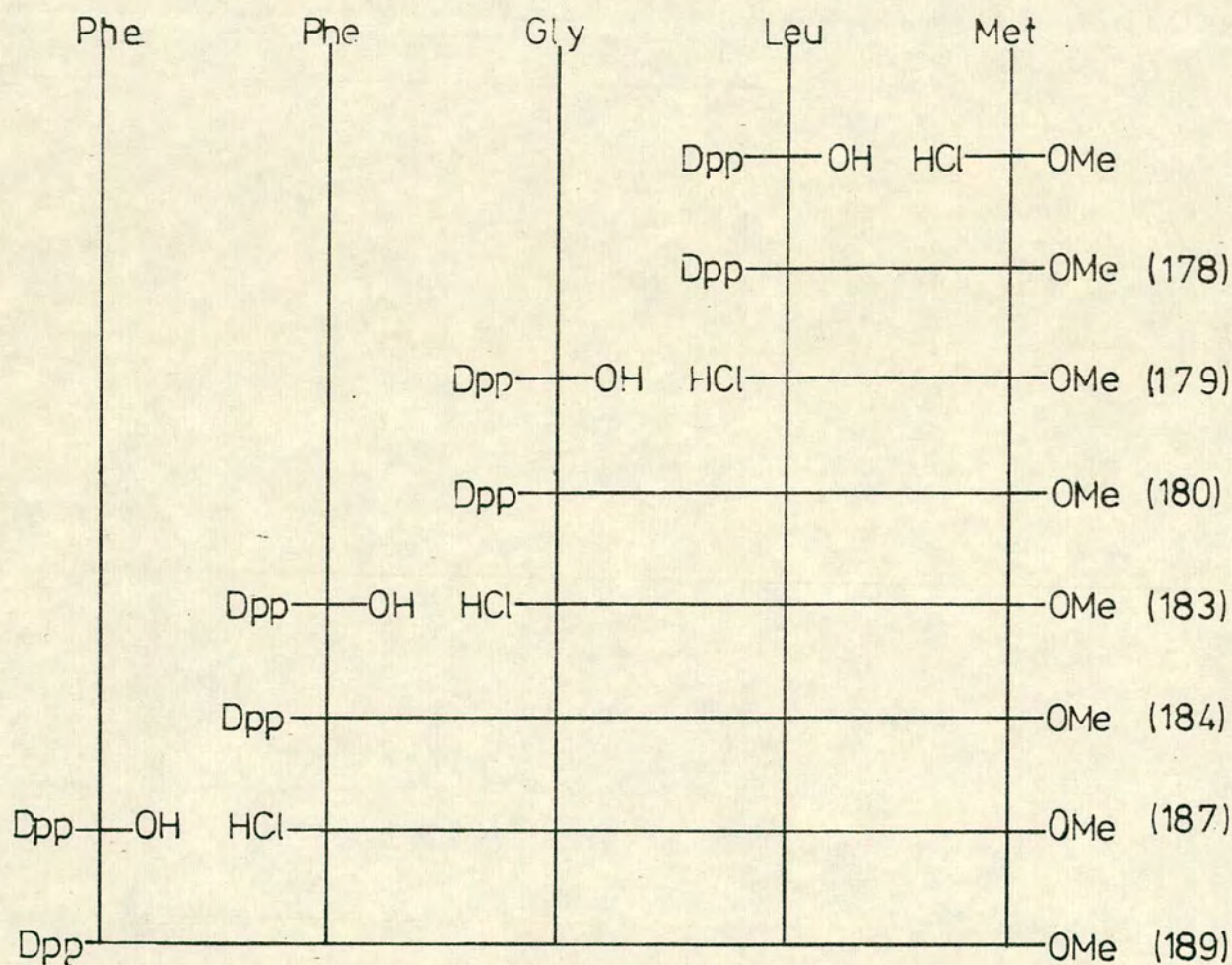


Fig.3.11

-20°C whereupon one equivalent of CptCl in methylene chloride and N-ethylmorpholine (NEM) were added dropwise. After fifteen minutes a solution of (165) in DMF and NEM are added and stirring was continued below -20°C for one hour and at ambient temperature for one hour. A few drops of water were added to destroy unreacted mixed anhydride and the solvent was removed. The residue was redissolved in a minimum of DMF and applied to a Sephadex LH20 gel filtration column (2.5x90cm) eluted with DMF. The product was isolated and passed through a second Sephadex LH20 column (1x50cm) again eluted with DMF. Using this procedure (172) and (173) were prepared in 22% and 26% yields respectively (*ca.* 70% based on recovered starting material). The addition of one equivalent of 2,6-lutidine increased the yield of (173) slightly from 26% to 31%. In comparison coupling of (171) to (165) or (177) using the DCCI method with HOBt as a catalyst gave the product in higher yields of 67–70% consistently but no starting material was recovered. Based on the results of other workers the coupling time was increased from one hour at room temperature to five hours. The resulting yield of (172) increased to 51% (69% based on recovered starting material). Further work is currently in progress evaluating and optimising conditions for fragment condensations of model compounds.

3.6.3. C-terminal Fragments of SP

In an attempt to observe structural conformations within a molecule by nmr and relate any observation to biological activity it was proposed to make the C-terminal fragments of SP from SP⁵⁻¹¹ to SP¹⁰⁻¹¹ with both amide and carboxyl termini. Fig.3.10 shows the list of fragments that have been made to date. The C-terminal fragments were prepared as shown in Fig.3.11. DppLeuOH (162) was coupled to HCl.MetOMe (92) in the usual manner to give DppLeu-MetOMe (178) in 80% yield after recrystallisation from ethyl acetate,

petroleum ether mixtures. Deprotection of (178) using a methanolic solution of hydrogen chloride took 2 hours at room temperature as shown by ^{31}P nmr. Removal of the solvent afforded HCl.Leu-MetOMe (179) as an oil which did not crystallise out from several solvent systems and so was washed with ether several times and used in the next coupling. The reaction of DppGlyOH (97) with (179) using standard conditions proved very troublesome. After aqueous workup DppGly-Leu-MetOMe (180) was isolated as a brown powder which would not crystallise. Initial attempts at purification involved gel filtration on Sephadex LH20 with methanol as the eluent, followed by droplet counter current chromatography using a MeOH, Buffer P, CHCl_3 , CCl_4 solvent system (Buffer P= 15ml. of acetic acid and 8.5g of ammonium acetate per 500ml of water) resulting in pure (180) in a 23% yield. A repeat experiment utilising dry flash chromatography for the purification step gave the product (180) in 51% yield. Treatment of (180) with 1.1 equivalents of NaOH in a dioxan water mixture removed the methyl ester in four hours to give DppGly-Leu-MetOH (181) (85%). (180) was stirred with a saturated methanolic ammonia solution overnight to give DppGly-Leu-MetNH₂ (182) (71%). Removal of the Dpp group from (180) gave the product HCl.Gly-Leu-MetOMe(183) as an oil which was washed with diethyl ether accordingly and used in the next coupling step immediately. Coupling of DppPheOH(93) to crude (183) afforded DppPhe-Gly-Leu-MetOMe (184) in a 71% yield after the usual aqueous work up and precipitation from ethyl acetate by light petroleum ether. Saponification of the methyl ester was complete after five hours to yield DppPhe-Gly-Leu-MetOH(185) (77%). Amidation of (184) using a saturated methanolic ammonia solution was complete after stirring overnight (as seen by tlc) to give DppPhe-Gly-Leu-MetNH₂ (186) in a 70% yield. Deprotection of (184) as previously described gave HCl.Phe-Gly-Leu-MetOMe (187) as a hygroscopic

white powder which proved difficult to handle. Hence it was immediately coupled to DppPheOH (93) under standard conditions to give DppPhe-Phe-Gly-Leu-MetOMe (188) (48%). The low yield might be due to using methylene chloride as a cosolvent and the use of DMF as the only solvent, which is known to give increased yields, might prove useful. Amidation, using the conditions previously mentioned, was complete after three days. The ammonia was replenished every twenty four hours by bubbling ammonia gas into the solution, cooled to -15°C , for 45 minutes. DppPhe-Phe-Gly-Leu-MetNH₂ (189) was obtained as a white powder in a 66% yield.

Since the full list of SP fragments and analogues has not yet been completed a detailed nmr study involving nOe experiments, solvent perturbation and determination of temperature coefficients is yet to be carried out. However, analysis of the proton nmr of the compounds made to date shows the NH- α CH coupling constants of all the amino acids except glycine to be 7.5–8.5Hz which is in direct agreement with reported NH- α CH J values of SP and C-terminal fragments^{158,192}. This is consistent with a σ value of -148° to -158° or from -84° to -94° . The vicinal coupling constants of the glycines' NH- α CH₂ is around 5.5Hz indicating an angle σ close to 180° . This also is in direct agreement with reported J values of glycines in SP and C-terminal fragments. However, using DMSO as the solvent ruptures any inter- or intramolecular hydrogen bonding therefore these figures may represent time averaged values derived from numerous constantly changing conformations. More work is required to ascertain the conformational properties of these compounds and to see if the presence of the Dpp group has an inductive effect on the tertiary structure.

AGONIST ACTIVITY

ANTAGONIST ACTIVITY

E.P.M.R.

BIOASSAY

		G.P.I.	R.C.	R.S.C.	G.P.I.	R.C.	R.S.C.
41530	Dpp. SP(7-11)	2,500	1,000	N.T.	N.T.	N.T.	N.T.
41531	Dpp. SP-(7.11)OMe.	>10,000	>300	N.T.	N.S.E.	N.S.E.	N.T.
41532	Dpp. [Trp ^{6,9}]SP-(6-11)	>10,000	>300	N.T.	N.S.E.	N.S.E.	N.T.
41533	Dpp. [Trp ^{6,9}]SP-(6-11).OMe	>10,000	>300	N.T.	N.S.E.	N.S.E.	N.T.
59673	Dpp.Phe.Phe.Gly.OMe.	>10,000	>300	>300	Non	N.S.E.	N.S.E.
59674	Dpp.Phe.Gly.Leu.Met.OH.	>10,000	>300	>300	Competitive	N.S.E.	N.T.
59675	Dpp.Gly.Leu.Met.NH ₂	>10,000	>300	>300	N.S.E.	N.S.E.	N.T.
59676	Dpp.Phe.Gly.Leu.Met.NH ₂	>10,000	partial	>300	N.S.E.	partial	N.S.E.
59677	pGlu-Gln.Phe.Phe.Gly.Leu.Met.Gly.Gly.OMe.	>10,000	agonist	300	N.S.E.	agonist	N.T.
39298	[pGlu ⁵]SP(5-11)	0.9	4.5	N.T.	N.S.E.	N.S.E.	N.T.
62037	[pGlu ⁵ ,Pro ¹⁰]SP-(5-11)	46.2	>300	5.8	N.T.	N.S.E.	N.T.
62038	[pGlu ⁵ ,Val ¹⁰]SP-(5-11)	1,632	>300	>300	N.T.	N.S.E.	N.S.E.
62039	Dpp.Gly.Leu.Met.Gly.Gly.OH.	>10,000	>300	>300	Non	N.S.E.	N.S.E.
62040	Dpp.Phe.Gly.Leu.Met.Gly.Gly.OH.	>10,000	>300	>300	Competitive	N.S.E.	N.S.E.
62041	pGlu.Gln.Phe.Phe.Gly.Leu.Leu.Gly.Gly.OMe.	>10,000	>300	>300	Non	N.S.E.	N.S.E.
56499	pGlu.Gln.Phe.Phe.Gly.Ile.Met.NH ₂	3,333	>300	83	Competitive	N.S.E.	N.S.E.
56500	HCl.H.Gln.Phe.Phe.Gly.OMe.	>10,000	>300	>300	N.S.E.	N.S.E.	N.S.E.
56501	pGlu.Gln.Phe.Phe.Gly.OH.	>10,000	>300	>300	N.S.E.	N.S.E.	N.S.E.

Table 1

3.6.4. Biological Activities

A list of analogues whose biological activities have been determined is given in Table 1. They were tested for *in vitro* agonist and antagonist activity on the guinea pig ileum (GPI), rat colon muscularis mucosae (RC) and rat spinal cord (RSC). These tissues are representative of the SP-P, SP-E and neuronal tachykinin receptors, respectively.

The Dpp C-terminal fragments of SP were devoid of any antagonistic activity and only Dpp-SP⁸⁻¹¹(186) showed some agonistic activity on the rat colon having a maximal effect which is only 20% of that achieved by SP. This activity in a small molecule may reflect differences in the binding requirements of the SP-P and SP-E receptors. Interestingly, Dpp-SP⁷⁻¹¹(189) did not show any agonistic activity since SP⁷⁻¹¹ has 20% activity of SP. This would suggest that the Dpp blocking group is preventing binding to the receptor site possibly due to an induced unfavourable conformation. If this is so biological testing of future analogues must include the deblocked as well as the blocked compound to ascertain the total effect of the Dpp group.

The importance of the C-terminal amide for agonist and antagonist activity is reinforced by the lack of activity of the SP extended analogues. The lack of activity of (172) and (172) may be due to either the -Gly-GlyOMe functionality disrupting the conformation at the active site, or that the tissues contain amidating enzymes and (172) and (173) are converted to (190) and (191) respectively and the -GlyNH₂ functionality causes disruption of the bioactive conformation at the active site. A clearer picture of the situation would be obtained from the synthesis and biological testing of (190), (191) and (192). The reported non-competitive antagonism of (167) and (170) in the GPI has been suggested to be because of a non-specific effect on the tissue rather than a

GlpGlnPhePheGlyLeuMetGlyNH₂ (190)

GlpGlnPhePheGlyLeuLeuGlyNH₂ (191)

GlpGlnPhePheGlyLeuLeuGlyNH₂ (192)

selective action at a tachykinin receptor

The results of these tests have shown that none of the analogues has any significant agonistic or antagonistic activity. However, whether any of the extended analogues interacted with the amidating enzyme cannot be determined from these experiments.

CHAPTER 4

EXPERIMENTAL

4.1. Notes

Melting points were taken in an open capillary tube on an electrically heated Buchi 510 melting point apparatus and are uncorrected. Optical rotations were measured on a AA1000 polarimeter using a 10cm cell. Thin-layer chromatography (tlc) was carried out on plastic sheets coated with silica gel 60GF-254 (Merck) in the following systems: (A) CHCl_3 -MeOH (9:1), (B) CHCl_3 -MeOH (+1% AcOH) (6:1), (C) CHCl_3 -MeOH (3:2), (D) nBuOH-AcOH- H_2O (3:1:1), (E) nBuOH-AcOH- H_2O (4:1:5), (F) EtOAc-Pyr-MeOH- H_2O (120:60:3:11). Visualisation of the compounds was achieved by a suitable combination of the following methods: iodine vapour, uv absorbtion at 254nm, ninhydrin for peptides with free amino groups and chlorine starch spray. High performance liquid chromatography (hplc) was carried out using a waters HPLC system ie. 2x6000A pumps, a Waters U6K injector, 680 gradient former, and a Waters uv detector (Model 441). Analytical separations were executed on an ODS 5 μ Hypersil column (3.9x300mm) and semi-preparative separations were carried out on an ODS 10 μ Hypersil column (7.8x300mm) using a gradient over 25 minutes, as specified in parentheses, between solvent A (0.05% TFA in water) and solvent B (0.05% TFA in acetonitrile). The flow rate was 1ml./min and elution of the samples was monitored at 254 and 229nm. Amino acid analyses were carried out on a LKB alpha machine following sealed tube hyrolyses at 110°C for 18 hours. Theoretical amino acid ratios are shown as a subscript following the amino acid in question. Infrared spectra were measured on a Perkin Elmer 781 specrophotometer. Ultraviolet spectra were measured in distilled methanol on a Pye-Unicam SP8-400 spectrophotometer. High resolution and low resolution fast atom bombardment (FAB) spectra were

measured on a Kratos MS50TC machine. Proton nmr spectra were recorded on a Bruker WH360 operating at 360MHz or a Bruker WP200 operating at 200MHz. Samples were dissolved in deuterated solvents indicated and tetramethylsilane (TMS) was used as an internal standard. Carbon-13 nmr spectra were measured on a Bruker WP200 machine operating at 50MHz. Samples were dissolved in deuterated solvents indicated and all chemical shifts were measured relative to TMS assigned at zero. Phosphorus-31 nmr spectra were recorded on a Jeol FX-60-Q machine operating at 24MHz. All chemical shifts were measured relative to 85% phosphoric acid assigned as zero. All solvents were distilled before use and the following were dried from the drying agent in parentheses; N,N-Dimethylformamide (DMF) (calcium hydride), ether refers to diethyl ether (sodium), dichloromethane (DCM) (calcium hydride). Dpp-amino acids⁶⁰, amino acid methyl esters, as their hydrochloride salt⁸¹ and HBr.Gly-GlyOMe⁷⁹ were prepared following literature methods.

4.2. Experimental

N-Diphenylphosphinylphenylalanylmethionine methyl ester (94)

DppPhe-MetOMe. To a stirred solution of DppPheOH (93) (2.19g, 6mM) in DCM (35ml.) cooled to 0°C were added NMM (0.66ml., 6mM) and a solution of diphenylphosphinyl chloride (1.42g, 6mM) in DCM (20ml.). After stirring for fifteen minutes a solution of HCl.MetOMe (92) (1.00g, 5mM) in DMF (10ml.) was added immediately followed by NMM (0.55ml., 5mM). The resulting mixture was stirred at low temperature for one hour before the cooling bath was removed and the temperature was allowed to rise to ambient when the reaction solvent was removed under reduced pressure to yield a colourless oil containing a white solid which was partitioned between ethyl acetate and water. The organic phase was washed with saturated NaHCO₃ solution (3x50ml.), water (1x50ml.), 5% citric acid solution (2x50ml.), water (1x50ml.), saturated NaHCO₃ solution

(2x50ml.), water (1x50ml.), brine (2x50ml.) and dried over anhydrous sodium sulphate. After filtering off the drying agent the organic solution was concentrated under reduced pressure and the desired product (94) was crystallised out by the addition of petrol (40–60) (2.1g, 82%), m.p. 155–157°C; tlc, $R_{f(A)}$ 0.6; (Found: C, 63.1; H, 6.0; N, 5.4. $C_{27}H_{31}N_2O_4PS$ requires C, 63.5; H, 6.1; N, 5.5%); $[\alpha]_D^{22}$ -69.7° (c 1.0 in MeOH); ν_{max} (CHBr₃ mull) 3360 3260 (N-H), 1740 (ester C=O), 1665 (amide C=O), 750 699cm⁻¹ (aromatic stretching); λ_{max} (MeOH) 207 (25,938), 220 (16,135) (sh), 264 (1,430), 272nm (919); δ_H (200MHz, CDCl₃) 2.0 (5H, m, Met β -CH₂), 2.5 (2H, tr, Met γ -CH₂), 3.2 (2H, m, Phe β -CH₂), 3.25 (1H, m, Phe α -CH), 3.75 (4H, m, OCH₃ and P-NH), 4.65 (1H, m, Met α -CH), 7.15–7.9 (15H, m, aromatic H's), 8.19 (1H, d, Met NH, $J_{NH-\alpha CH}$ 7.7Hz); δ_C (50MHz; CDCl₃) 15.1 (S-CH₃), 29.9 (Met γ -C), 31.0 (Met β -C), 39.3 39.5 (d, Phe β -C J_{P-C} 6.1Hz), 51.9 (α -C), 52.1 (O-CH₃), 56.2 (α -C), 126.7–137.0 (aromatic C's), 171.8 172.0 (C=O's); Amino acid analysis, Phe₁, Met₁; hplc, R_t 19.1 mins (20–100%B):

N-Diphenylphosphinylglycylphenylalanylmethionine methyl ester (98)

DppGly-Phe-MetOMe. To a stirred solution of DppPhe-MetOMe (94) (11.2g, 23mM) in methanol was added a methanolic solution of hydrogen chloride (138mM, 6eq.). ³¹P nmr showed the reaction to be complete after two hours whereupon the solvent was removed and the residue was washed with ether (3x50ml.) to remove the DppOMe (96) byproduct. The resulting HCl.Phe-MetOMe (95) was stored in a dessicator over phosphorus pentoxide until needed.

DppGlyOH (97) (6.5g, 23mM) was dissolved in DCM (200ml.) and cooled to -5°C with stirring. NMM (2.5ml., 23mM) and a solution of DppCl (5.4g, 23mM) in DCM (10.2ml.) were added dropwise maintaining the low temperature and the mixture was stirred for fifteen minutes. HCl.Phe-MetOMe (95) (prepared as above) in DMF (100ml.), NMM (2.5ml., 23mM) and 2,6-lutidine (2.67ml., 23mM)

were added dropwise and the mixture was stirred for one hour below 0°C and one hour at ambient temperature. After the removal of the reaction solvent the resulting oil was partitioned between ethyl acetate and water and was worked up as for (94). The drying agent was filtered from the solution which was concentrated and the product was triturated under hexane. Recrystallisation from CHCl_3 and hexane yielded (98) as a white powder (9.0g, 70%), m.p. 143–144°C; tlc, $R_{f(A)}$ 0.55; (Found: C, 61.32; H, 6.0; N, 7.4. $\text{C}_{29}\text{H}_{34}\text{N}_3\text{N}_5\text{PS}$ requires C, 61.4; H, 6.0; N, 7.4%); $[\alpha]_{\text{D}}^{22}$ –26.1°; (c 1 in MeOH); ν_{max} (CHBr_3 mull) 3240 3200 (N–H), 1750 (ester C=O), 1660 (amide C=O's), 750 695 (aromatic stretches); λ_{max} (MeOH) 203 (51,556), 259 (2,111), 265 (2,389), 272nm (1,778); δ_{H} (200MHz, CDCl_3) 2.0 (5H, m, S– CH_3 and Met β – CH_2), 2.4 (2H, tr, Met γ – CH_2), 3.1 (2H, d, Phe β – CH_2), 3.55 (2H, m, Gly α – CH_2), 3.6 (3H, s, OCH_3), 4.7 (1H, m, P–NH), 4.6 (1H, m, Met α –CH), 4.8 (1H, m, Phe α –CH), 7.1–7.9 (16h, m, aromatic H's and Met–NH), 8.7 (1H, d, Phe–NH); δ_{C} (50MHz, CDCl_3) 15.1 (S– CH_3), 29.9 (Met γ –C), 31.1 (Met β –C), 37.6 (Phe β –C), 44.6 (Gly α –C), 51.6 (α –C), 52.0 (O– CH_3), 54.4 (α –C), 126.6–136.6 (aromatic C's), 170.1 170.8 171.8 (C=O's); δ_{P} (24MHz, CDCl_3) 25.1; m/z 567 492 376 257. HRMS 567.1950 ($[M+H]^+$); Amino acid analysis, Gly₁0.99, Met₁0.44, Phe₁1.01 ; ; hplc, R_t 21.4 mins. (0–100%B):

Glycylphenylalanylmethionine methyl ester hydrochloride. (99)

HCl.Gly–Phe–MetOMe. (98) (11g, 19.4mM) was dissolved in a methanolic hydrogen chloride solution (29ml., 116.3mM) and stirred at 35–40°C. ^{31}P nmr and tlc showed the reaction to be complete within 30 minutes. The solvent was removed under reduced pressure and the residue was taken up in a little methanol. The desired product (99) was obtained by trituration with anhydrous ether as a white powder (6.74g, 86%), m.p. 176–177°C; tlc, $R_{f(E)}$ 0.18; (Found: C, 49.7; H, 6.5; N, 10.3. $\text{C}_{14}\text{H}_{28}\text{N}_3\text{O}_4\text{SCl}$ plus 0.5mol of H_2O requires C, 49.5; H, 6.6; N, 10.2%); $[\alpha]_{\text{D}}^{22}$ –8.4° (c 1 in MeOH); ν_{max} (CHBr_3 mull) 3500–2500 (NH_3^+), 3300

3200 (N-H's), 1740 (ester C=O), 1660 (amide C=O's), 745 695cm^{-1} (aromatic stretches); λ_{max} (MeOH) 203nm (17,280); δ_{H} (200MHz, $d_6\text{DMSO}$) 1.95 (2H, m, Met g[b]-CH₂), 2.05 (3H, s, S-CH₃), 2.5 (2H, m, Met γ -CH₂), 3.9 (2H, m, Phe β -CH₂), 3.45 (2H, q, Gly α -CH₂), 3.65 (3H, s, O-CH₃), 4.4 (1H, m, Met α -CH), 4.65 (1H, m, Phe α -CH), 7.25 (5H, m, Phe's aromatic H's), 8.0 (3H, br.s, NH₃⁺), 8.65 (1H, d, Met N-H, $J_{\text{NH}-\alpha\text{CH}}7.8$), 8.7 (1H, d, Phe N-H, $J_{\text{NH}-\alpha\text{CH}}8.7$); δ_{C} (50MHz, $d_6\text{DMSO}$) 14.5 (S-CH₃), 29.5 (Met γ -C), 30.3 (Met β -C), 37.6 (Phe β -C), 39.9 (Gly α -C), 51.1 (α -C), 51.8 (O-CH₃), 54.0 (α -C), 126-137 (aromatic C's), 165.5 170.8 171.8 (C=O's); m/z 369 165. HRMS 369.1722 ($[M-C]^{+}$); Amino acid analysis, Gly₁1.00, Met₁0.51, Phe₁1.00;

N-Diphenylphosphinylglycylglycylphenylalanylmethionine methyl ester (100)
DppGly-Gly-Phe-MetOMe. DppGlyOH (97) (5.2g, 19.25mM) and NMM (2.12ml., 19.25mM) were dissolved in DMF (20ml.) and in an ice-salt cooling bath to -5°C . DppCl (4.55g, 19.25mM) in DCM (14.2ml.) was added dropwise and the mixture was stirred for fifteen minutes below 0°C . A chilled solution of (99) (6.94g, 17.2mM) in DMF (50ml.), NMM (1.9ml., 17.2mM) and 2,6-lutidine (2.24ml., 19.25mM) were added dropwise. Stirring was continued for one hour at 0°C and one hour at ambient temperature. The reaction mixture was worked up using the same procedure as for (94) to yield the product (100) as a white powder which would not crystallise from numerous solvent systems. Trace contaminants were removed by dry flash silica chromatography using 5% MeOH in CHCl₃ as the eluant (6.5g, 60%), m.p. $168-169^{\circ}\text{C}$; tlc, $R_{\text{f(A)}}0.5$; $[\alpha]_{\text{D}}^{22} -23.5^{\circ}$ (c 1 in MeOH); ν_{max} (CHBr₃ mull) 3300-3200 (N-H's), 1740 (ester C=O), 1660 (amide C=O's), 750 700cm^{-1} (aromatic stretches); λ_{max} (MeOH) 208 (41,980), 259 (1499), 265 (1874), 272nm (1374); δ_{H} (200MHz, CDCl₃) 1.95 (5H, m, Met β CH₂ and S-CH₃), 2.3 (2H, tr, Met γ CH₂), 3.05 (2H, m, Phe β CH₂), 3.6 (5H, m, O-CH₃ and Gly¹ α CH₂), 3.9 (2H, m, Gly² α CH₂), 4.55 (2H, m, Met α CH and

P-NH), 4.8 (1H, m, Phe α C-H), 7.0–7.9 (16H, m, Phe NH and aromatic H's), 8.05 (1H, tr, Gly² N-H, $J_{\text{NH}-\alpha\text{CH}}$ 5.2), 8.1 (1H, d, Met N-H, $J_{\text{NH}-\alpha\text{CH}}$ 7.8); δ_{C} (50MHz, CDCl₃) 15.1 (S-CH₃), 29.9 (Met γ C), 31.0 (Met β -C), 37.9 (Phe β -C), 43.1 43.9 (Gly α -C's), 51.4 54.4 (Phe and Met α -C's), 52.1 (O-CH₃), 126.5–136.7 (aromatic C's), 168.7 170.9 171.1 172.0 (C=O's); δ_{P} (24MHz, CDCl₃) 27.6; m/z 625 315 258 201. HRMS 625.2250 ($[M+H]^+$); Amino acid analysis, Gly₂2.01, Met₁0.74. Phe₁1.00; hplc, R_t 20.6mins (0–100%B, 25mins):

Bis-N,O-Diphenylphosphinyl tyrosine methyl ester (102) DppTyr(Dpp)OMe. A solution of DppCl (40.8g, 172.7mM) in DCM (90ml.) and NMM (28.5ml., 259mM) was added to a suspension of HCl.TyrOMe (20g, 86.3mM) in DCM (75ml.) cooled to 0°C. After stirring at low temperature for one hour and at room temperature for one hour the solvent was removed under reduced pressure and the residue was partitioned between chloroform and water. The organic phase was washed with saturated NaHCO₃ solution (3x75ml.), water (1x75ml.), 5% citric acid solution (2x75ml.), water (1x75ml.), saturated NaHCO₃ solution (2x75ml.), water (1x75ml.), cold 0.25M NaOH solution (2x75ml.), water (1x75ml.), brine (2x75ml.) and dried over anhydrous sodium sulphate. Removal of the drying agent and concentration of the solution under reduced pressure precipitated the crude product which was recrystallised from chloroform petrol(40–60) mixtures to yield pure DppTyr(Dpp)OMe (102) (43.3g, 85%), m.p. 184–186°C (Lit¹⁶⁸. 189–190°C); tlc, $R_{f(A)}$ 0.6; $[\alpha]_D^{22}$ –20.3 (c 1 in MeOH); ν_{max} (CHBr₃ mull) 3380 (N-H), 1740 (ester C=O), 750 695cm^{–1} (aromatic stretches); λ_{max} (MeOH) 207 (49,812), 223 (45,481), 260 (3,898), 267 (4,548), 274nm (3,465); δ_{H} (200MHz, CDCl₃) 2.95 (2H, d, β -CH₂, $J_{\alpha\text{CH}-\beta\text{CH}}$ 6.1Hz), 3.55 (4H, m, O-CH₃ and α -CH), 3.95 (1H, m, P-NH), 7.05 (4H, ABq, Tyr aromatic H's), 7.2–7.9 (24H, m, Dpp aromatic H's); δ_{C} (50MHz, CDCl₃) 40.38 40.46 (d, β -C, $J_{\text{P}-\beta\text{C}}$ 4.1Hz), 51.9 (O-CH₃), 54.6 (α -C), 120.6–133.4 (aromatic C's), 149.9 150.1 (d, Tyr C₄, $J_{\text{P}-\text{C}}$ 8.2Hz), 172.8 173.0 (C=O,

J_{P-C} 5.7Hz); δ_P (24MHz, $CDCl_3$) 23.3 (P-NH), 30.6 (P-O); hplc, R_t 23 mins (0-100%B):

N-Diphenylphosphinyl tyrosine dicyclohexylamine salt (103) DppTyrOH.DCHA.
 DppTyr(Dpp)OMe (102) (5.96g, 10mM) was dissolved in peroxide free 1,4-dioxane (70ml.) and 1M sodium hydroxide solution (25ml.) was added. The resulting clear, pale yellow solution was stirred for at room temperature for five hours when tlc showed the reaction to be complete. The dioxane was removed under reduced pressure and the resulting aqueous solution was acidified to pH 3 with saturated citric acid precipitating out of solution a white gum. This gum was extracted into ethyl acetate (3x30ml.), the organic solution was washed with water (2x20ml.), brine (2x20ml.) and dried over anhydrous sodium sulphate. After removing the drying agent the solvent was evaporated off under reduced pressure to leave a white foam which was dissolved in ethyl acetate (10ml.) and dicyclohexylamine (2.5ml., 12mM) was added causing white crystals to deposit immediately. These were filtered and recrystallised from methanol ether mixtures to yield DppTyrOH.DCHA (103) (4.22g, 75%), m.p.218-220°C (Lit. 218-220°C); (Found: C, 70.4; H, 7.7; N, 4.9. $C_{33}H_{43}N_2O_4P$ requires C, 70.4; H, 7.7; N, 5.0%); $[\alpha]_D^{22} +14.2^\circ$ (c 1 in MeOH); ν_{max} (CHBr₃ mull) 3500-2800 ($-NH_2^+$), 3160 (N-H), 2940 2860 (aliphatic C-H's), 1570 (CO_2^-), 745 $695cm^{-1}$ (aromatic stretches); δ_H (200MHz, d_6DMSO) 1.0-2.0 (2H, m, DCHA CH_2 's), 2.65-3.00 (4H, m, $\beta-CH_2$ and DCHA CH's), 3.4 (1H, m, $\alpha-CH$), 4.8 (1H, m, P-NH), 6.8 (4H, q, Tyr aromatic H's), 7.3-7.8 (10H, m, Dpp aromatic H's), 9.2 (1H, br.s, phenolic -OH); δ_C (50MHz, d_6DMSO) 24.2 25.2 (DCHA C's), 30.5 ($\beta-C$), 52.1 (O- CH_3), 56.0 ($\alpha-C$), 114.4-135.4 (aromatic C's), 155.5 (Tyr C_4), 164.1 (C=O); δ_P (24MHz, d_6DMSO) 25.0;

N-Diphenylphosphinyl tyrosine dihydrate (105) DppTyrOH.H₂O

DppTyrOH.DCHA (103) (2.63g, 4.67mM) was suspended in ethyl acetate and an equal volume of citric acid was added and the mixture was shaken until all the solid had dissolved. The organic solution was then washed with saturated citric acid (2x20ml.) and water (3x20ml.) before drying over anhydrous sodium sulphate. After removing the drying agent the solution was concentrated to half volume under reduced pressure and hexane was added dropwise until the solution became cloudy whereupon it was cooled to yield DppTyrOH.2H₂O as a white crystalline solid (1.75g, 90%), m.p. 85–86°C; tlc, $R_{f(D)}$ 0.2; (Found: C, 60.1; H, 5.8; N, 3.4; C₂₁H₂₄NO₆P (includes 2 moles of H₂O) requires C, 60.4, H, 5.8, N, 3.4%); $[\alpha]_D^{22}$ –151° (*c* 1 in MeOH); ν_{\max} (CHBr₃ mull) 3600–2200 (–CO₂H), 3260 (N–H), 1745 (acid C=O), 750 700cm^{–1} (aromatic stretches); λ_{\max} (MeOH) 204 (23,003), 225 (24,116), 265 (2,597), 273nm (2,579); δ_H (200MHz, d₆DMSO) 2.8 (2H, m, β -CH₂), 3.75 (1H, m, α -CH), 3.9 (1H, m, P–NH), 6.7 (4H, q, Tyr aromatic H's), 7.05–7.70 (10H, m, Dpp aromatic H's); δ_C (50MHz, d₆DMSO) 38.9 39.0 (d, β -C, J_{P-C} 6.3Hz), 55.4 (α -C), 115.1–133.7 (aromatic C's), 156.2 (Tyr C₄), 174.0 (C=O); *m/z* 381.1130 (*M*⁺); hplc, R_t 17.6 mins (0–100%B, 25mins):

Diphenylphosphinyl phenyl ester (112) DppOPh To a stirred solution of phenol (0.94g, 10mM) and NMM (1.1ml., 10mM) in DCM (10ml.) cooled to –5°C was added a solution of DppCl (2.36g, 10mM) in DCM (10ml.) dropwise so as to maintain the low temperature. Stirring was continued below 0°C for one hour and at ambient temperature for one hour after which the solvent was removed under reduced pressure and the residue was worked up as for (102). After removing the drying agent the solution was concentrated under reduced pressure and hexane was added dropwise until the resulting cloudiness persisted. Cooling resulted in the desired product crystallising out as white needle shaped crystals. These were filtered and dried over phosphorus pentoxide m.p. 137–138°C; tlc, $R_{f(A)}$ 0.60; ν_{\max} (CHBr₃ mull) 3380 3360

(aromatic C-H's), 1590 (aromatic C=C), 1440 (P-Ph), 770 750 730 700 cm^{-1} (aromatic stretches); λ_{max} (MeOH) 208 (20,110), 223 (15,406), 264 (1,646), 272nm (1,176); δ_{H} (80MHz, CDCl_3) 6.9–8.2 (m, aromatic H's); δ_{P} (24MHz, CDCl_3) 30.4;

N-Diphenylphosphinyltyrosylglycylglycylphenylalanylmethionine methyl ester (108) DppTyr-Gly-Gly-Phe-MetOMe. DppGly-Gly-Phe-MetOMe (100) (6.4g, 10.25mM) was dissolved in a methanolic hydrogen chloride solution and stirred at 35–40°C. ^{31}P nmr and tlc showed the reaction to be complete after half an hour whereupon the solvent was removed under reduced pressure and the residue was washed with dry ether several times. DppTyrOH.2H₂O (105) (4.7g, 11.28mM) was dissolved in DMF (30ml.) and left over 4A molecular sieves for three hours. The solution was decanted and cooled to –5°C in an ice salt bath and NMM (1.24ml., 11.28mM) and DppCl (2.67g, 11.28mM) in DCM (5ml.) were added dropwise. Stirring was continued for fifteen minutes when a chilled solution of HCl.Gly-Gly-Phe-MetOMe (prepared as above) in DMF (30ml.) was added followed by NMM (1.13ml., 10.25mM) and 2,6-lutidine (1.31ml., 10.28mM). After stirring for one hour at 0°C and one hour at room temperature the reaction solvent was removed under reduced pressure and the residue was worked up as for (94). The dried organic solution was concentrated under reduced pressure and cooled to –20°C to yield the desired product as a white powder (4.9g, 61%), m.p. 116–118°C; tlc, $R_{\text{f(A)}}$ 0.5; (Found: C, 61.0; H, 5.9; N, 8.9. $\text{C}_{40}\text{H}_{46}\text{N}_5\text{O}_8\text{PS}$ plus 1.5mol of water requires C, 60.0; H, 6.1; N, 8.6%); $[\alpha]_{\text{D}}^{22}$ –60.5° (c 1 in MeOH); ν_{max} (CHBr_3 mull) 3300 (br, N-H), 1740 (ester C=O), 1655 (amide C=O), 750 695 cm^{-1} (aromatic stretches); λ_{max} (MeOH) 213 (23,493), 225 (21,010), 266 (2,101), 273nm (2,149); δ_{H} (360MHz, CDCl_3) 1.9 (2H, m, Met β -CH₂), 2.0 (3H, s, Met S-CH₃), 2.3 (2H, m, Met γ -CH₂), 2.95 (2H, m, Tyr β -CH₂), 3.1 (2H, m, Phe β -CH₂), 3.6–3.9 (8H, m, Gly's α -CH₂'s, Tyr α -CH and O-CH₃), 4.35 (1H, m, Met α -CH), 4.65 (1H, m, Phe α -CH), 4.9 (1H, m, P-NH), 6.8 (4H, ABq, Tyr aromatic

H's), 7–7.75 (13H, m, NH's and aromatic H's), 8.45 (1H, br.s, Gly N-H); δ_C (50MHz, $CDCl_3$) 15.1 (S-CH₃), 29.7 (Met γ -C), 31.0 (Met β -C), 37.8 (Phe β -C), 39.0 (Tyr β -C), 43.1 43.7 (Gly α -C's), 51.6 (α -C), 52.1 (OCH₃), 54.6 (α -C), 57.0 (Tyr α -C), 115.6–136.8 (aromatic C's), 155.9 (Tyr C₄), 169.5 170.1 171.3 171.8 174.4 (C=O's); δ_P (24MHz, $CDCl_3$) 21.4; m/z (FAB) 788 (M^+), 478, 421, 336 and 201. HRMS 788.2883 ($[M+H]^+$); Amino acid analysis, Gly₂2.15, Met₁0.48, Tyr₁0.95, Phe₁0.98; hplc, R_t 17.2 mins (20–100%B):

N-Diphenyltyrosylglycylglycylphenylalanylmethionine (115)

DppTyr-Gly-Gly-Phe-MetOH. (108) (2g, 2.54mM) was dissolved in DMF (10ml.) and 1M NaOH solution (5.3ml., 5.3mM) was added with stirring. Stirring continued for six hours when tlc showed the saponification to be complete. The solvent was removed under reduced pressure at room temperature and the residue was taken up in some water (5ml.) and acidified to pH 3 with saturated citric acid solution. The resulting white gum was extracted with ethyl acetate (3x15ml.) washed with water (2x10ml.), brine (2x10ml.) and dried over anhydrous sodium sulphate. was obtained by trituration with petrol (40–60), After removal of the drying agent the solution was concentrated and the product (115) (1.78g, 91%), tlc R_{f(B)}0.5; $[\alpha]_D^{22}$ -54.0 (c 1 in MeOH); ν_{max} (CHBr₃ mull) 3650–2700 (COOH), 3300 (N-H), 1725 (acid C=O), 1660 (amide C=O), 750 700cm⁻¹ (aromatic stretches); λ_{max} (MeOH) 211 (22,751), 225 (17,799), 267 (1,857), 274nm (2,012); δ_H (360MHz, d₆DMSO) 1.9 (2H, m, Met β -CH₂), 2.0 (3H, s, S-CH₃), 2.45 (2H, m, Met γ -CH₂), 2.8 (2H, m, Tyr β -CH₂), 2.95 (2H, m, Phe β -CH₂), 3.45 (1H, m, Tyr α -CH), 3.6–3.85 (4H, m, Gly α -CH₂'s), 4.2 (1H, m, Met α -CH), 4.55 (1H, m, Phe α -CH), 5.9 (1H, m, P-NH), 6.9 (4H, q, Tyr aromatic H's), 7.1–7.7 (10H, m, Dpp aromatic H's), 7.9 (1H, d, Phe N-H, $J_{NH-\alpha CH}$ 8.5Hz), 8.3 (1H, d, Met N-H, $J_{NH-\alpha CH}$ 7.7Hz), 8.6 (1H, tr, Gly N-H, $J_{NH-\alpha CH}$ 5.8Hz), 8.7 (1H, tr, Gly N-H, $J_{NH-\alpha CH}$ 5.9Hz); δ_C (50MHz, d₆DMSO) 14.7 (S-CH₃), 29.8 (Met γ -C), 30.8 (Met

β -C), 37.7 (Phe β -C), 38.4 (Tyr β -C), 42.0, 42.8 (Gly α -C), 51.3 (α -C), 53.8 (α -C), 57.3 (α -C), 57.3 (Tyr α -C), 115–137 (aromatic C's), 156.2 (Tyr C₄), 168.7, 169.5, 171.3, 173.1, 173.7 (C=O's); m/z 775, 478, 421, 336, 201. HRMS 774.2726 ($[M+H]^+$); Amino acid analysis, Tyr₁0.92, Gly₂2.04, Phe₁1.06, Met₁1.00; hplc, R_t 19.0 mins (0–100%B, 25mins):

Tyrosylglycylglycylphenylalanylmethionine (116) Tyr–Gly–Gly–Phe–Met. (115) (774mg, 1mM) was dissolved in a 2M solution of hydrogen chloride in a 2:1 mixture of water and peroxide free dioxane (6ml., 12eq.). ³¹P nmr showed the deprotection to be complete after six hours. The solution was lyophilised and the solid was redissolved in a minimum of 5% aqueous acetic acid and applied to a Sephadex G15 gel filtration column eluted with 5% acetic acid to yield 361mg (63%) of product with a minor impurity. This was easily removed either by reverse phase semi-preparative hplc or by partition chromatography on Sephadex G25 using a nBuOH:AcOH:H₂O (4:1:5) solvent system (220mg, 26%), m.p. 190°C; tlc, R_{f(E)} 0.43; (Found: C, 55.4; H, 6.2; N, 11.8. C₂₇H₃₅N₅O₇S plus 0.5mol of acetic acid requires C, 55.7; H, 6.2, N, 11.6 %); $[\alpha]_D^{22}$ –20.8° (*c* 0.5, H₂O); ν_{\max} (CHBr₃ mull) 3600–2500 (NH₃⁺), 3300 (N–H), 1750–1630 (br.C=O); λ_{\max} (MeOH) 196 (42,452); δ_H (360MHz, d₆DMSO) 1.9 (2H, m, Met β -CH₂), 2.05 (3H, s, Met S-CH₃), 2.50 (2H, m, Met γ -CH₂), 2.85 (2H, m, Tyr β -CH₂), 2.95 (2H, m, Phe β -CH₂), 3.6–3.9 (4H, m, Gly α -CH₂'s), 4.0 (1H, br.s, Tyr α -CH), 4.35 (1H, m, Met α -CH), 4.6 (1H, m, Phe α -CH), 6.9 (4H, ABq, Tyr aromatic H's), 7.2 (5H, m, Phe aromatic H's), 8.1 (5H, br.s, d, tr, NH₃⁺, Phe N–H and Gly N–H), 8.38 (1H, d, Met N–H, J_{NH- α CH} 7.8Hz), 8.77 (1H, tr, Gly N–H, J_{NH- α CH} 5.6Hz); m/z 574, 185. HRMS 574.2335 ($[M+H]^+$); Amino acid analysis, Tyr₁0.95, Gly₂2.01, Phe₁1.05, Met₁1.01; hplc, R_t 21.4 mins (0–60%B):

N-Diphenylphosphinyl methionyl glycyl glycine methyl ester (160)

DppMet-Gly-GlyOMe. DppMetOH (158) (10g, 0.029mM) was dissolved in DCM (250ml.) and cooled to -5°C whereupon NMM (3.19ml., 0.029mM) and DppCl (6.8g, 0.029mM) in DCM (24.2ml.) was added dropwise so as to maintain the low temperature. After fifteen minutes of stirring HBr.Gly-GlyOMe (159) (6.0g, 0.026mM) in DMF (40ml.) was added dropwise followed by NMM (2.9ml., 0.026M) and the reaction mixture was stirred below 0°C for one hour and allowed to rise to ambient temperature before removing the solvent and working up the residue as for (94). The product was obtained slightly impure and was further purified by dry flash silica chromatography using 5% MeOH in CHCl_3 as the eluant to yield DppMet-Gly-GlyOMe (160) as a white foam (11.2g, 93%), tlc, $R_{f(A)}$ 0.4; ν_{max} (CHBr_3 mull) 3440 3260 (N-H), 1740 (ester C=O), 1670 (amide C=O); λ_{max} (MeOH) 203 (18,719), 224 (11,365), 264 (1,242), 271nm (955); δ_{H} (200MHz, CDCl_3) 1.95 (5H, m, S-CH₃ and Met β -CH₂), 2.45 (2H, m, Met γ -CH₂), 3.6 (4H, m, O-CH₃ and Met α -CH), 3.85 (4H, m, Gly's α -CH₂'s), 4.65 (1H, m, P-NH), 7.25-7.85 (10H, m, aromatic H's), 7.9 (1H, tr, Gly N-H), 8.65 (1H, tr, Gly N-H); δ_{C} (50MHz, CDCl_3) 14.8 (S-CH₃), 29.9 (Met γ -C), 33.0 (Met β -C), 40.7 42.5 (Gly's α -C's), 54.1 (Met α -C), 128.3-132.7 (aromatic C's), 169.8 170.0 173.0 (C=O's); δ_{P} (24MHz, CDCl_3) 25.1; m/z 478 304 256 201. HRMS 478.1565 ($[M+H]^+$); Amino acid analysis, Met₁ 0.40, Gly₂ 2.00; hplc, R_t 18.3 mins (0-100%B):

Methionylglycylglycyl methyl ester hydrochloride (161) HCl.Met-Gly-GlyOMe

DppMet-Gly-GlyOMe (160) (10g, 21mM) was dissolved in a minimum of methanol and a methanolic solution of hydrogen chloride (40ml., 126mM) was added with stirring. The reaction mixture was warmed to $35-40^{\circ}\text{C}$ in a water bath and the progress was monitored by ^{31}P nmr and tlc which showed it to be complete after 20 minutes. The solvent was removed and the residue was triturated with ether to give a white powder. This was recrystallised from methanol ether mixtures to yield HCl.Met-Gly-GlyOMe (161) (5.6g, 85%), m.p.

109–111°C; tlc, $R_{f(d)}$ 0.36; $[\alpha]_D^{22} +23^\circ$ (c 1 in MeOH); ν_{\max} (CHBr₃ mull) 3600–2400 (NH₃⁺), 3340 3205 (N–H), 1745 (ester C=O), 1655 (amide C=O's), 750 700cm⁻¹ (aromatic stretches); λ_{\max} (MeOH) 205 (15,314); δ_H (200MHz, d₆DMSO) 2.05 (5H, m, S–CH₃ and Met β -CH₂), 2.55 (2H, m, Met γ -CH₂), 3.65 (3H, s, O–CH₃), 3.9 (5H, m, Gly α ' α -CH₂ and Met α -CH), 8.5 (3H, br.s, NH₃⁺), 8.6 (1H, tr, Gly N–H), 9.0 (1H, tr, Gly N–H); δ_C (50MHz, d₆DMSO) 14.4 (S–CH₃), 28.3 (Met γ -C), 30.8 (Met β -C), 40.9 41.8 (Gly α ' α -C's), 51.7 (O–CH₃ and Met α -C), 168.5 168.8 170.1 (C=O's); m/z 279 ([*M*-C]⁺, 100%), 218, 190, 148. HRMS 278.1174 ([*M*-C]⁺); Amino acid analysis, Gly₂2.00, Met₁0.34; hplc, R_t 4.2 mins (20–100%B):

N-Diphenylphosphinylleucylmethionylglycylglycine methyl ester (163) Dpp Leu-Met-Gly-GlyOMe. DppLeuOH (162) (6.0g, 18mM) was dissolved in dry dichloromethane (200ml.) and cooled to -5°C with stirring. NMM (1.98ml., 18mM) and a solution of DppCl (4.26g, 18mM) in dichloromethane (32ml.) was added dropwise so as to maintain the low temperature. Stirring was continued for 15 minutes afterwhich HCl.Met-Gly-GlyOMe (161) (5.0g, 16) dissolved in DMF (35ml.) was added dropwise followed by NMM (1.76ml., 16mM). The reaction mixture was stirred for one hour at -5°C and one hour at ambient temperature. The solvent was removed and the residue was worked up following the same procedure as for (94) yielding the desired product slightly impure. The trace impurities were removed by dry flash silica chromatography using 5% MeOH in CHCl₃ as the eluant to give DppLeu-Met-Gly-GlyOMe (163) as a white powder. Attempted recrystallisations yielded only a gelatinous precipitate (7.5g, 70%), m.p.191–192°C; tlc, $R_{f(A)}$ 0.47; (Found: C, 57.0; H, 6.8; N, 9.5. C₂₈H₃₉N₄O₆PS requires C, 56.9; H, 6.7, N, 9.5%); $[\alpha]_D^{22} -60.4^\circ$ (c 1 in MeOH); ν_{\max} (CHBr₃ mull) 3280 3240 (N–H), 1750 (ester C=O), 1670 1640 (amide C=O's), 750 695cm⁻¹ (aromatic stretches); λ_{\max} (EtOH) 260 (1,100), 268 (1,416), 271nm (1,130); δ_H (360MHz, CDCl₃) 0.84 (6H, q, Leu δ -CH₃'s), 1.7 (2H, m, Leu β -CH₂),

1.8 (1H, m, Leu γ -CH₂), 2.05 (3H, s, Met S-CH₃), 2.3 (2H, m, Met β -CH₂), 2.55 (2H, m, Met γ -CH₂), 3.5 (1H, m, Leu α -CH), 3.55 (3H, s, O-CH₃), 3.75 (2H, m, Gly α -CH₂), 4.2 (2H, m, Gly α -CH₂), 4.25 (1H, m, Met α -CH), 4.5 (1H, m, P-NH), 7.3–7.75 (12H, m, aromatic H's and 2NH's), 9.1 (1H, tr, Gly NH); δ_C (20MHz, CDCl₃) 15.0 (S-CH₃), 21.8 22.6 (Leu δ -C's), 24.5 (Leu γ -C), 29.9 (Met γ -C), 30.4 (Met β -C), 40.6 43.0 (Glys' α -C's), 43.3 43.7 (d, Leu β -C, J_{P-C} 8.8Hz), 51.8 (O-CH₃), 53.3 53.9 (α -C's), 127.3–134.7 (aromatic C's), 169.9 171.5 174.5 (C=O's); δ_P (24MHz, CDCl₃) 24.4; m/z 591.5468 ($[M+H]^+$); Amino acid analysis, Gly₂2.02, Met₁0.55, Leu₁0.99; hplc, R_t 16.7 mins (20–100%B):

N-Diphenylphosphinylleucylmethionylglycylglycine (164)

DppLeu-Met-Gly-GlyOH. (163) (500mg, 0.85mM) was suspended in dioxane (5ml.) at room temperature and a 1M NaOH solution (0.85ml., 0.85mM) was added. After three minutes a clear solution was formed and after seven hours the reaction was finished as seen on tlc. The dioxane was removed under reduced pressure and the resulting aqueous solution was acidified to pH 3 with saturated citric acid solution. A white gum precipitated out and was extracted into ethyl acetate (3x20ml.). The organic solution was washed with water (2x20ml.), brine (2x20ml.) and dried over anhydrous sodium sulphate. After filtering off the drying agent the solution was concentrated and cooled to yield DppLeu-Met-Gly-GlyOH (164) as a white flocculant powder. The product was filtered and dried (409mg, 85%), m.p. 189°C; tlc, R_{f(z)}0.23; (Found: C, 54.7; H, 6.5; N, 9.3. C₂₇H₃₉N₄O₇PS (includes one mol of H₂O) requires C, 54.5; H, 6.6; N, 9.4%); $[\alpha]_D^{22}$ -61.1° (c 1 in MeOH); ν_{max} (CHBr₃ mull) 3500–2600 (CO₂H), 3340 3260 (NH's), 1780 (acid C=O), 1660 (amide C=O's), 750 700cm⁻¹ (aromatic stretches); λ_{max} (MeOH) 203 (34,138), 222 (15,801), 261 (1,615), 271nm (1,153); δ_H (360MHz, d₆DMSO) 0.77 (6H, q, Leu δ -CH₃), 1.5 (2H, m, Leu β -CH₂), 2.0 (3H, m, Leu γ -CH and Met β -CH₂), 2.0 (3H, s, S-CH₃), 2.3 (2H, m, Met γ -CH₂), 3.3

(1H, m, Leu α -CH obscured by the water signal), 3.7 (2H, d, Gly α -CH₂), 3.79 (2H, dq, Gly α -CH₂), 4.3 (1H, m, Met α -CH), 5.6 (1h, m, P-NH), 7.5–7.85 (10H, m, aromatic H's), 8.0 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.6Hz), 8.14 (1H, d, Met NH, $J_{\text{NH}-\alpha\text{CH}}$ 7.7Hz), 8.75 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.5Hz); δ_{c} (50MHz, d₆DMSO) 14.6 (S-CH₃), 21.6 22.9 (Leu δ -C's), 23.9 (Leu γ -C), 29.6 (Met γ -C), 31.3 (Met β -C), 40.6 42.0 (Glys' α -C's), 43.1 (Leu β -C), 51.8 53.6 (α -C's), 128.4–133.5 (aromatic C's), 169.1 171.0 171.4 173.7 (C=O's); δ_{p} (24MHz, d₆DMSO) 22.1; m/z 557 286 201. HRMS 577.2250 ($[M+H]^+$); Amino acid analysis, Gly₂1.95, Met₁0.85, Leu₁1.03; hplc, R_t 15.2 mins (20–100%B):

N-Diphenylphosphinylglycylleucylmethionylglycylglycine methyl ester (166)
DppGly-Leu-Met-Gly-GlyOMe. To a stirred solution of (163) (500mg, 0.85mM) in methanol (2ml.) was added a solution of hydrogen chloride in methanol (45ml., 5.1mM). Stirring was continued until tlc and ³¹P nmr showed the reaction to be complete two hours later. The solvent was removed under reduced pressure and the residue was washed with dry ether several times and stored in a dessicator over phosphorus pentoxide until needed. DppGlyOH (465mg, 1.69mM) was stirred in DMF (5ml.) and cooled to -5°C when NMM (0.186ml., 1.69mM) and a solution of DppCl (400mg, 1.69mM) in DCM (1.92ml.) were added dropwise. The mixture was stirred <0°C for fifteen minutes and then a solution of HCl.Leu-Met-Gly-GlyOMe (722mg, 1.69mM) in DMF (2ml.) , NMM (0.186 ml., 1.69mM) and 2,6-lutidine (0.196ml., 1.69mM) were added. Stirring was continued for one hour at 0°C and one hour at room temperature. The solvent was removed under reduced pressure and the residue taken up in a minimum of fresh DMF and applied to a gel filtration column (2.5x80cm) packed with Sephadex LH20 and eluted with DMF. The product was obtained (650mg, 60%) slightly impure and was further purified by wet flash silica chromatography using 5% MeOH in CHCl₃ as the eluant to yield

DppGly-Leu-Met-Gly-GlyOMe as a white powder (596mg, 55%), m.p. 216–217°C; tlc, $R_{f(A)}$ 0.29; (Found: C, 55.2; H, 6.6; N, 10.6. $C_{30}H_{42}N_5O_7PS$ requires C, 55.6; H, 6.5; N, 10.8%); $[\alpha]_D^{22}$ –28.7 (c 1 in MeOH); ν_{max} (CHBr₃ mull) 3280 3240 (NH's), 1750 (ester C=O), 1680 1660 1610 (amide C=O's), 750 730 700cm⁻¹ (aromatic stretches); λ_{max} (MeOH) 207 (24,873), 225 (14,509), 267 (1,160), 274 (777); δ_H (200MHz, d₆DMSO) 0.85 (6H, dd, Leu δ -CH₃'S), 1.55 (3H, m, LEU B-CH₂ AND I-CH), 2.0 (5H, m, S-CH₃ and Met β -CH₂), 2.5 (2H, m, Met γ -CH₂), 3.4 (2H, m, Gly α -CH₂), 3.75 3.85 (4H, d's, Gly α CH₂'s), 4.4 (2H, m, Leu and Met α -CH's), 5.8 (1H, m, P-NH), 7.4–7.9 (10H, m, aromatic H's), 8.1–8.5 (4H, m, NH's); δ_C (50MHz, d₆DMSO) 14.7 (S-CH₃), 21.6 23.2 (Leu δ -C's), 24.2 (Leu γ -C's), 29.7 (Met γ -C), 31.5 (Met β -C), 40.4 40.7 41.7 (Glys' α -C's), 43.4 (Leu β -C), 51.1 52.2 (α -C's), 51.6 (O-CH₃), 128.7–134.0 (aromatic C's), 169.3 170.2 171.5 172.3 (C=O's); δ_P (24MHz, d₆DMSO) 23.5; Amino acid analysis, Gly₃3.00, Met₁0.54, Leu₁1.00; hplc, R{t} 20.1 mins (20–100%B):

N-Diphenylphosphinylglycylleucylmethionylglycylglycine (167)

DppGly-Leu-Met-Gly-GlyOH. To a stirred suspension of (166) (100mg, 0.154mM) in peroxide free dioxane (2ml.) was added 1M NaOH solution (0.16ml., 0.16 mM). The mixture was stirred for thirty minutes when tlc showed the reaction to be complete. The dioxane was removed under reduced pressure and the aqueous mixture was acidified to pH 3 with saturated citric acid. The water was removed and the residue redissolved in a minimum of DMF and applied a gel filtration column (1x50cm) packed with Sephadex LH20 and eluted with DMF. Fractions 25–28 were pooled and the solvent removed under reduced pressure to leave a residue which when triturated with ether yielded the desired product as a white powder (60mg, 61%), m.p. 171–172°C; tlc, $R_{f(z)}$ 0.19; $[\alpha]_D^{22}$ –30.0° (c 1 in DMF); ν_{max} (CHBr₃ mull) 3280 (NH), 1740 (ester C=O), 1640 (amide C=O), 750 695cm⁻¹ (aromatic stretches); λ_{max} (MeOH) 205 (30,798), 221

(20,095), 266 (1,458), 277nm (1,204); δ_H (360MHz, d_6 DMSO) 0.85 (6H, q, Leu δ -CH₃'s), 1.5 (3H, m, Leu β -CH₂ and γ -CH), 1.9 (2H, m, Met β -CH₂), 1.95 (3H, s, S-CH₃), 2.5 (2H, m, Met γ -CH₂), 3.3 (2H, m, Gly¹ α -CH₂ obscured by the water signal), 3.8 (4H, m, Gly^{4,5} α -CH₂'s), 4.5 (2H, m, Leu and Met α -CH's), 5.7 (1H, m, P-NH), 7.4-7.9 (10H, m, aromatic H's), 8.1 (5H, m, Gly NH's and Leu or Met NH), 8.3 (1H, d, Leu or Met NH, $J_{NH-\alpha CH}$ 7.8Hz); m/z 634 258 230 201. HRMS 634.2464 ($[M+H]^+$); Amino acid analysis, Gly₃2.96, Met₁0.96, Leu₁1.01; hplc, R_t 23.5mins (0-100%B):

N-Diphenylphosphinylphenylalanylglycylleucylmethionylglycylglycine methyl ester (169) DppPhe-Gly-Leu-Met-Gly-GlyOMe. (166) (735mg, 1.13mM) was dissolved in a minimum of methanol and a solution of hydrogen chloride in methanol (3.4ml., 6.8mM) was added and the mixture was warmed to 35-40°C with stirring. ³¹Pnmr and tlc showed the reaction to be complete after 30 minutes. The solvent was removed under reduced pressure and the residue was washed with ether several times and stored in a dessicator until needed. DppPheOH (170) (413mg, 1.13) was dissolved in dichloromethane (5ml.) and cooled to -5°C whereupon NMM (124 μ l., 1.13mM) and a solution of DppCl (267mg, 1.13mM) in dichloromethane (0.45ml.) was added dropwise. Stirring was continued for fifteen minutes and then a solution of HCl.Gly-Leu-Met-Gly-GlyOMe (168) (1.13mM) in DMF (5ml.) was added dropwise followed by NMM 124 μ l., 1.13mM) and 2,6-lutidine (131 μ l., 1.13mM). The reaction mixture was stirred for one hour at 0°C and one hour at room temperature after which the solvent was removed under reduced pressure and the residue was worked up in the same manner as for (94). Concentration of the dried organic solution and cooling led to the crystallisation of the desired product which was then filtered and dried (652mg, 68%), m.p. 172-174°C; tlc, $R_{f(A)}$ 0.45; (Found: C, 58.9; H, 6.5; N, 10.5. C₃₉H₅₁N₆O₈PS requires C, 58.8; H, 6.5; N, 10.2%); $[\alpha]_D^{22}$ -46.9° (c 1

in MeOH); ν_{\max} (CHBr₃) 3320 (NH), 1750 (ester C=O), 1660 (amide C=O's), 750 695cm⁻¹ (aromatic stretches); λ_{\max} (MeOH) 206 (21,622), 227 (9,539), 268 (1,272), 274nm (954); δ_{H} (360MHz, d₆DMSO) 0.82 (6H, q, Leu δ -CH₃'s), 1.7 (3H, m, Leu γ -CH and β -CH₂), 2.0 (3H, s, S-CH₃), 2.05 (2H, m, Met β -CH₂), 2.3 (2H, m, Met γ -CH₂), 2.9 (2H, m, Phe β -CH₂), 3.5 (1H, m, Phe α -CH), 3.6 (3H, s, O-CH₃), 3.65-3.9 (6H, m, Gly α -CH₂'s), 4.4 (2H, m, Leu and Met α -CH's), 6.0 (1H, m, P-NH), 7.1-7.7 (15H, m, aromatic H's), 7.96 (1H, d, Leu or Met NH, $J_{\text{NH}-\alpha\text{CH}}$ 7.9), 8.1 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.8Hz), 8.3 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.9), 8.7 (1H, d, Leu or Met NH, $J_{\text{NH}-\alpha\text{CH}}$ 8.5Hz), 8.6 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.9Hz); (60mg, 61%), δ_{C} (50MHz, d₆DMSO) 14.6 (S-CH₃), 21.4 22.9 (Leu δ -C's), 29.5 (Met γ -C), 31.7 (Met β -C), 38.5 38.7 (Phe β -C, $J_{\text{P-C}}$ 10.4Hz), 40.3 40.4 41.7 (Gly α -C's), 42.5 (Leu β -C), 51.6 51.8 (α -C's), 51.9 (O-CH₃), 56.9 (α -C), 126.4-138.1 (aromatic C's), 169.0 169.2 170.0 171.2 172.3 173.6 (C=O's); δ_{P} (24MHz, d₆DMSO) 21.5; Amino acid analysis, Phe₁1.01, Gly₃3.10, Leu₁0.96, Met₁0.66; hplc, R_t 19.4mins (20-100%B):

N-Diphenylphosphinylphenylalanylglycylleucylmethionylglycylglycine (170)

DppPhe-Gly-Leu-Met-Gly-GlyOH. To a stirred suspension of (169) (250mg, 0.32mM) in peroxide free dioxane (2ml.) was added 1M NaOH solution (0.35ml., 0.35mM). Stirring was continued for 4 hours when tlc showed the reaction to be complete. The dioxane was removed under reduced pressure and the residue was worked up in the same manner used for (163) to yield DppPhe-Gly-Leu-Met-Gly-GlyOH (170) as a white powder (166mg, 68%), m.p.225-227°C; tlc, R_{f(B)}0.22; $[\alpha]_{\text{D}}^{22}$ -31.9° (*c* 1 in MeOH); ν_{\max} (CHBr₃ mull) 3600-2800 (CO₂H), 3340 3280 (NH), 1740 (acid C=O), 1640 (amide C=O's), 750 690cm⁻¹ (aromatic stretches); λ_{\max} (MeOH) 209 (59,348), 270 (10,620), 276nm (9,995); δ_{H} (360MHz, d₆DMSO) 0.8 (6H, dd, Leu δ -CH₃'s), 1.5-1.9 3H, m, Leu β -CH₂ and γ -CH), 1.95 (3H, s, S-CH₃), 2.0 (2H, m, Met β -CH₂), 2.45 (2H, m, Met

γ -CH₂), 2.95 (2H, d, Phe β -CH₂), 3.5 (1H, m, Phe α -CH), 3.6–3.9 (6H, m, Gly α -CH₂'s), 4.4 (2H, m, Leu and Met α -CH₂'s), 6.1 (1H, m, P-NH), 7.1–7.3 (15H, m, aromatic H's), 8.0 (1H, d, Leu or Met NH, $J_{\text{NH}-\alpha\text{CH}}$ 8.0Hz), 8.05 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.9Hz), 8.15 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.8Hz), 8.6 (1H, d, Leu or Met NH, $J_{\text{NH}-\alpha\text{CH}}$ 8.4Hz), 8.7 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.7Hz); m/z 803 320 201. HRMS 781.3148 ($[M+H]^+$); Amino acid analysis, Phe₁1.04, Gly₃2.82, Leu₁1.03, Met₁1.01; hplc, R_t 18.1 mins (20–100%B):

N-Diphenylphosphinylleucylglycylglycine methyl ester (174)

DppLeu-Gly-GlyOMe. To a stirred solution of DppLeuOH (2.3g, 6.9mM) in dry dichloromethane (20ml.) at -5°C was added NMM (0.76ml., 6.9mM) and a solution of DppCl (1.63g, 6.9mM) in dichloromethane (3.5ml.) dropwise. Stirring was continued for fifteen minutes after which a solution of HBr.Gly-GlyOMe (1.4g, 6.3mM) in DMF (10ml.), NMM (0.69ml., 6.3mM) and 2,6-lutidine (0.79ml., 6.9mM) were added dropwise. The reaction mixture was stirred for one hour below 0°C and one hour at ambient temperature. The solvent was removed under reduced pressure and the residue was worked up following the same procedure as for (94). DppLeu-Gly-GlyOMe was obtained as an off white foam which was slightly impure as seen on tlc. The trace impurities were removed by wet flash silica chromatography using 5% MeOH in CHCl₃ as the eluant to yield the product (174) as a white foam (2.5g, 85%), tlc, $R_{f(A)}$ 0.36; ν_{max} (CHBr₃ mull) 3260 (NH's), 1750 (ester C=O), 1660 (amide C=O's), 750 695cm⁻¹ (aromatic stretches); λ_{max} (MeOH) 205 (22,607), 225 (14,520), 267 (1,678), 276nm (1,011); δ_{H} (200MHz, CDCl₃) 0.75 (6H, q, Leu δ -CH₃'s), 1.55 (2H, dd, Leu β -CH₂), 1.7 (1H, m, Leu γ -CH), 3.45 (1H, m, Leu α -CH), 3.55 (3H, s, O-CH₃), 3.9 (4H, m, Gly α -CH₂'s), 4.3 (1H, m, P-NH), 7.3–7.9 (11H, m, aromatic H's and Gly NH), 8.95 (1H, tr, Gly NH); δ_{C} (50MHz, CDCl₃) 21.8 22.7 (Leu δ -C's), 24.4 (Leu γ -C), 40.9 42.8 (Gly α -C's), 43.3 43.5 (d, Leu β -C, $J_{\text{P-C}}$ 9.2Hz), 51.8 (O-CH₃), 53.8 (Leu α -C), 128.3–133.2

(aromatic C's), 170.1 170.2 174.0 (C=O's); δ_P (24MHz, $CDCl_3$) 24.7; m/z 460.2001 ($[M+H]^+$); hplc, R_t 15.7 mins (20–100%B):

Leucyl glycylglycine methyl ester hydrochloride (175) HCl.Leu-Gly-GlyOMe.

A solution of (174) (1.1g, 2.4mM) and hydrogen chloride (14.4mM) in methanol was stirred at 40°C for thirty minutes when the reaction was shown to be complete by tlc and ^{31}P nmr. The solvent was removed under reduced pressure yielding a white solid which was washed with ether and recrystallised from methanol ether mixtures. The product was filtered off and dried to yield hygroscopic white crystals (454mg, 64%), m.p. 99–101°C; tlc $R_{f(D)}$ 0.61; λ_{max} (MeOH) 204 (5,235); δ_H (200MHz, d_6DMSO) 0.9 (6H, dd, Leu $\delta-CH_3$'s), 1.7 (3H, m, Leu $\beta-CH_2$ and $\gamma-CH$), 3.4 (1H, br.s, Leu $\alpha-CH$), 3.6 (3H, s, O- CH_3), 3.8 (4H, m, Gly $\alpha-CH_2$'s), 8.45 (3H, br.s, NH_3^+), 8.6 (1H, tr, Gly NH, $J_{NH-\alpha CH}$ 5.8Hz), 9.05 (1H, tr, Gly NH, $J_{NH-\alpha CH}$ 5.6Hz); δ_C (50MHz, d_6DMSO) 21.9 22.0 (Leu $\delta-C$'s), 23.2 (Leu $\gamma-C$), 39.9 (Leu $\beta-C$), 40.4 41.8 (Gly $\alpha-C$'s), 50.7 (Leu $\alpha-C$), 51.2 (O- CH_3), 168.3 168.9 169.6 (C=O's); m/z 260.1610 ($[M-C]^+$); hplc, R_t 3.0mins (20–100%B):

N-Diphenylphosphinylleucylleucylglycylglycine methyl ester (176)

DppLeu-Leu-Gly-GlyOMe. To a stirred solution of DppLeuOH (739mg, 2.2mM) in dry dichloromethane (25ml.) at -5°C was added NMM (0.25ml., 2.2mM) and a solution of DppCl (520mg, 2.2mM) in dry dichloromethane (0.6ml.) dropwise so as to maintain the low temperature. After stirring for fifteen minutes HCl.Leu-Gly-GlyOMe (175) (600mg, 2.0mM), NMM (0.22ml., 2.0mM) and 2,6-lutidine (0.26ml., 2.2mM) were added dropwise and stirring was continued for one hour below 0°C and one hour at ambient temperature. The solvent was removed under reduced pressure and the residue was worked up in the same manner as for (94) to yield crude (178). The product was further purified either by gel filtration on Sephadex LH20 with MeOH as the eluant or by wet flash

silica chromatography using 5% MeOH in CHCl_3 as the eluant. (176) was obtained as a white powder which refused to crystallise in various solvent systems (871mg, 76%), m.p. 187–188°C; tlc, $R_{f(A)}$ 0.40; (Found: C, 60.5; H, 7.3; N, 9.6; P, 5.5. $\text{C}_{29}\text{H}_{41}\text{N}_4\text{O}_6\text{P}$ requires C, 60.8; H, 7.2; N, 9.8; P, 5.4%); ν_{\max} (CHBr_3 mull) 3280 (NH), 1745 (ester C=O), 1680 1660 (amide C=O's), 700 730 cm^{-1} (aromatic stretches); λ_{\max} (EtOH) 222 (15,866), 264 (1,291), 271nm (922); δ_{H} (200MHz, CDCl_3) 0.85 (12H, m, Leu δ - CH_3 's), 1.7 (6H, m, Leu β - CH_2 's and γ -CH's), 3.55 (4H, s, m, O- CH_3 and Leu α -CH), 3.9 (4H, m, Gly α - CH_2 's), 4.2 (2H, m, P-NH and Leu α -CH), 7.1–7.95 (12H, m, aromatic h's, Gly NH and Leu NH), 8.95 (1H, tr, NH); δ_{C} (20MHz, CDCl_3) 21.4 21.9 22.8 23.1 (Leu δ -C's), 24.7 25.0 (Leu γ -C's), 39.5 40.7 (Gly α -C's), 43.2 43.7 (Leu β -C's), 51.9 (O- CH_3), 53.2 54.3 (Leu α -C's); δ_{P} (24MHz, CDCl_3) 24.5; Amino acid analysis, Gly₂2.02, Leu₂1.98; hplc, R_t 17.6 mins (20–100%B):

***pyro*-Glutamylglutamylphenylalanylphenylalanylglycylleucylmethionylglycylglycine methyl ester (172) pGln-Gln-Phe-Phe-Gly-Leu-Met-Gly-GlyOMe.**

Method A: HCl.Leu-Met-Gly-GlyOMe (165) was prepared by stirring (164) (112mg, 0.2mM) for one hour with a methanolic solution of hydrogen chloride (0.6ml., 1.2mM). The reaction was followed by ^{31}P nmr and tlc. The solvent was removed under reduced pressure and the resulting brown oil was washed with ether and dried under vacuum over phosphorus pentoxide. pGln-Gln-Phe-Phe-GlyOH (171) (122mg, 0.2mM) was stirred in DMF (2ml.) and cooled to -25°C . NMM (22 μl ., 0.2mM) and CptCl (27.7mg, 0.2mM) in DCM (80 μl) was added dropwise so as to maintain the low temperature. The solution was stirred for fifteen minutes after which a solution of (165) (0.2mM) in DMF (4ml.) to which NEM (25 μl ., 0.2mM) had been added immediately before, was added. Stirring was continued for one hour below -20°C and one hour at room

temperature. The solvent was removed under reduced pressure and the residual oil was taken up in a minimum of DMF and applied to a chromatography column packed with Sephadex LH20 and eluted with DMF. The product was obtained slightly impure and applied to a second gel filtration column (1x50cm) eluted with DMF from which it was obtained in a pure form (43mg, 22%).

Method B. The title compound was prepared in the same manner as described in method A except that the reaction was allowed to stir at room temperature for five hours. The work up procedure was identical to yield (172) as a white powder (100mg, 51%).

Method C. (165) (0.2mM) was prepared as described in method A and stored in a dessicator over phosphorus pentoxide until needed. (200) (122mg, 0.2mM) and N-hydroxybenzotriazole (27.5mg, 0.4mM) were dissolved in DMF (4ml.) and cooled to 0°C. A solution of DCCI (80mg, 0.4mM) in DMF (1ml.) was added dropwise to the stirred solution. Ten minutes later a solution of (166) (0.2mM) in DMF (2ml.) and NMM (22μl. 0.2mM) was added and stirring was continued for 2 hours at 0°C and 22 hours at ambient temperature. The reaction mixture was worked up following the same procedure used in method A to yield the (172) (256mg, 67%), δ_H (360MHz, d_6 DMSO) 0.85 (6H, dd, Leu δ -CH₃'s), 1.5 (2H, dd, Leu β -CH₂), 1.65 1.80 (4H, m, Gln β -CH₂), 1.8 (1H, m, Leu γ -CH), 1.95 (1H, m, Met β -CH₂), 2.0 (3H, s, S-CH₃), 2.1 (4H, m, Gln γ -CH₂), 2.45 (2H, m, Met γ -CH₂), 2.8 2.95 (4H, m, Phe β -CH₂'s), 3.6 (3H, s, O-CH₃), 3.7 (4H, m, Gly α -CH₂'s), 3.85 (2H, d, Gly α -CH₂), 4.05 (1H, m, pGln α -CH), 4.2 (1H, m, Gln α -CH), 4.3 (2H, m, Leu and Met α -CH' s), 4.5 (2H, m, Phe α -CH's), 6.75 (1H, br.s, Gln 1ry amide NH), 7.1-7.25 (10H, m, aromatic H's), 7.8 (1H, s, pGln NH), 8.05 (1H, d, Leu NH), 8.1-8.4 (7H, m, NH's); m/z 981.4504 ($[M+H]^+$); Amino acid analysis, Gln₂2.03, Gly₃2.95, Met₁0.60, Leu₁1.03, Phe₂1.95; hplc, R_t 11.4mins (0-100%B):

pyroGlutamylglutamylphenylalanylphenylalanylglycylleucylleucylglycylglycine
(175) pGln-Gln-Phe-Phe-Gly-Leu-Leu-Gly-GlyOMe.

Method A. (176) (94mg, 0.16mM) was dissolved in methanol (1ml.) and a solution of hydrogen chloride in methanol (0.30ml., 1.0mM) was added. After stirring for two hours at room temperature ^{31}P nmr and tlc showed the reaction to be complete. The solvent was removed and the residue was washed with ether several times and stored in a dessicator over phosphorus pentoxide until needed. (173) (100mg, 0.16mM) was dissolved in DMF (10ml.) and cooled to -20°C . NEM (18 μl ., 0.16mM) and CptCl (23mg, 0.16mM) in DCM (92 μl .) were added dropwise maintaining the low temperature. Stirring was continued for fifteen minutes when a solution of (177) (0.16mM) in DMF (1ml.) was added dropwise. This was followed by NEM (21 μl ., 0.16mM) and stirring was continued for one hour below 0°C and for one hour at room temperature. The reaction mixture was worked up in the manner as for (172) to yield (173) as a white powder (41mg, 26%).

Method B. (173) was prepared following the same procedure as outlined in method A except that 2,6-lutidine (19 μl ., 0.16mM) was added after adding the final aliquot of NEM (53mg, 34%).

Method C. (177) (0.16mM) was prepared following the same procedure outlined in method A and stored in a dessicator over phosphorus pentoxide until needed. (173) (100mg, 0.16mM) and N-hydroxybenzotriazole (22mg, 0.32mM) were dissolved in DMF (4ml.) and cooled to 0°C . A solution of DCCI (64mg, 0.32mM) in DMF (1ml.) was added dropwise and the reaction mixture was stirred for ten minutes. A solution of (177) (0.16mM) in DMF (2ml.) and NMM (18 μl ., 0.16mM) were added to the mixture and stirring was continued for 2 hours at 0°C and 22 hours at room temperature. The reaction mixture was

worked up following the same procedure as for (172) to yield (173) as a white powder (107mg, 68%), δ_H (360MHz, d_6 DMSO) 0.85 (12H, m, Leu δ -CH₃'s), 1.45 (4H, m, Leu β -CH₂'s), 1.65 1.80 (4H, m, Gln β -CH₂'s), 2.1 (4H, m, Gln γ -CH₂'s), 2.85 2.95 (4H, m, Phe β -CH₂'s), 3.65 (3H, s, O-CH₃), 3.7 (4H, m, Gly α -CH₂'s), 3.85 (2H, m, Gly α -CH₂), 4.0 (1H, m, pGln α -CH), 4.2 (1H, m, Gln α -CH), 4.25 4.35 (2H, m, Leu α -CH's), 4.5 4.55 (2H, m, Phe α -CH's), 6.8 (1H, br.s, Gln 1ry amide NH), 7.1-7.25 (10H, m, aromatic H's), 7.75 (1H, s, pGln NH), 7.92 (1H, d, Leu NH, $J_{NH-\alpha CH}$ 8.2Hz), 7.97 (1H, d, Phe NH, $J_{NH-\alpha CH}$ 8.2Hz), 8.01 (1H, d, Gln NH, $J_{NH-\alpha CH}$ 8.5Hz), 8.04 (1H, d, Leu NH, $J_{NH-\alpha CH}$ 8.4Hz), 8.11 (1H, tr, Gly NH), 8.2 (3H, m, Phe NH and 2xGly NH's); m/z 964 217. HRMS 963.4940 ($[M+H]^+$); Amino acid analysis, Gln₂2.13, Gly₃2.91, Leu₂1.91, Phe₂2.06; hplc, R_t 15.2mins (20-100%B):

N-Diphenylphosphinylleucylmethionine methyl ester (178) DppLeu-MetOMe.

To a stirred solution of DppLeuOH (10g, 30.2mM) in dry DCM (50ml.) cooled to -5°C was added NMM (3.3ml., 30.2mM) and a solution of DppCl (7.1g, 30.2mM) in dry DCM (35.5ml.) dropwise so as to maintain the low temperature. Stirring was continued for fifteen minutes when a solution of HCl.MetOMe (5.5g, 27.5mM) in DMF (20ml.) was added followed by NMM (3.0ml., 27.5mM). The reaction mixture was stirred for one hour at -5°C and one hour at room temperature. The solvent was removed and the residual oil was worked up in the same manner as for (94). The desired product was obtained slightly impure and so was recrystallised from ethyl acetate petrol (40-60) mixtures (10.5g, 80%), m.p. 165-167°C; tlc, $R_{f(A)}$ 0.64; (Found: C, 60.2; H, 5.8; N, 6.9. C₂₄H₃₃N₂O₄PS requires C, 60.5; H, 5.9; N, 7.0%); $[\alpha]_D^{22}$ -49.8° (c 1 in MeOH); ν_{max} (CHBr₃) 3360 3250 (NH), 1740 (ester C=O), 1665 (amide C=O's), 750 695nm (aromatic stretches); λ_{max} (MeOH) 205 (39,270), 220 (20,970), 265 (1,525), 273nm (1,144); δ_H (200MHz, CDCl₃) 0.80 (6H, dd, Leu δ -CH₃'s), 1.5 (1H, m, Leu γ -CH), 1.75 (2H, m, Leu β -CH₂), 2.05 (5H, m, S-CH₃ and Met β -CH₂), 2.5 (2H, dd, Met

γ -CH₂), 3.6 (2H, m, P-NH and Leu α -CH), 3.75 (3H, s, O-CH₃), 4.6 (1H, m, Met α -CH), 7.3-7.9 (10H, m, aromatic H's); δ_C (50MHz, CDCl₃) 15.2 (S-CH₃), 21.9 22.6 (Leu δ -C's), 24.4 (Leu γ -C), 30.0 31.2 (Met β and γ -C's), 43.5 43.7 (d, Leu β -C, J_{P-C} 5.7Hz), 51.7 53.1 (α -C's), 52.1 (O-CH₃), 128.3-133.3 (aromatic C's), 172.0 173.2 (C=O's); δ_P (24MHz, CDCl₃) 24.8; Amino acid analysis, Leu₁1.00, Met₁0.51; hplc, R_t 17.8 mins (0-100%B):

N-Diphenylphosphinylglycylleucylmethionine (180) DppGly-Leu-MetOMe.

(174) (877mg, 1.84mM) was dissolved in a minimum of methanol and a methanolic solution of hydrogen chloride (4.8ml., 11mM) was added. The solution was stirred for thirty minutes at 35-40°C when ³¹P nmr and tlc showed the reaction to be complete. The solvent was removed under reduced pressure and the residue was washed with ether several times and dried in a vacuum over phosphorus pentoxide. DppGlyOH (506mg, 1.84mM) was dissolved in dry dichloromethane and cooled to -5°C. NMM (0.2ml., 1.84mM) and a solution of DppCl (435mg, 1.84mM) in dry DCM (5.3ml.) were added dropwise and the reaction mixture was left to stir for fifteen minutes. A solution of HCl.Leu-MetOMe (179) (1.84mM) in DMF (2ml.) and NMM (0.20ml.1.84mM) were then added and the reaction was left for a further one hour at -5°C and one hour at room temperature. The solvent was removed and the residual oil was worked up as for (94). The resulting crude product was initially purified by gel filtration on Sephadex LH20 with MeOH as the eluant followed by droplet counter current chromatography using nBuOH:AcOH:H₂O (4:1:5) solvent system but poor yields of only 23% (270mg) were obtained. A repeat experiment purified the product on a dry flash column over silica eluting with 5% MeOH in CHCl₃ to the product pure in final yield of 51% (501mg), m.p. 156°C; tlc, R_{f(A)}0.60; (Found C,58.1; H, 6.7; N, 7.9. C₂₆H₃₆N₃O₅PS requires C, 58.5; H, 6.8; N, 7.9%); [α]_D²² -35.1° (c 1 in MeOH); ν_{max} (CHBr₃ mull) 3350 3230 (NH), 1740 (ester

C=O), 1660 (amide C=O's), 750 695 cm^{-1} (aromatic stretches); λ_{max} (MeOH) 204 (20,065), 224 (14,088), 264 (3,415), 272nm (2,988); δ_{H} (360MHz, CDCl_3) 0.9 (6H, dd, Leu δ -CH₃'s), 1.7 (3H, m, Leu β -CH₂ and γ -CH), 2.05 (5H, m, S-CH₃ and Met β -CH₂), 2.5 (2H, dd, Met γ CH₂), 3.4 (5H, m, O-CH₃ and Gly α -CH₂), 3.95 (1H, q, P-NH), 4.55 (1H, m, Met α -CH), 4.65 (1H, m, Leu α -CH), 7.35 (1h, d, Leu NH, $J_{\text{NH}-\alpha\text{-CH}}$ 7.9Hz), 7.45–7.9 (10H, m, aromatic H's), 8.0 (1H, d, Met NH, $J_{\text{NH}-\alpha\text{CH}}$ 8.1Hz); δ_{C} (50MHz, CDCl_3) 15.3 (S-CH₃), 21.9 22.8 (Leu δ -C's), 24.9 (Leu γ -C), 30.1 31.6 (Met β and γ -C's), 128.6–132.5 (aromatic C's), 170.4 171.9 172.1 (C=O's); δ_{P} (24MHz, CDCl_3) 27.2; m/z 534 343 258 201. HRMS 534.2191 ($[M+H]^+$); Amino acid analysis, Gly₁0.98, Leu₁1.02, Met₁0.57; hplc, R_t 16.8 mins (0–100%B):

N-Diphenylphosphinylglycylleucylmethionine (183) DppGly-Leu-MetOH.

(180) (1g, 1.84mM) was suspended in peroxide free dioxane (20ml.) and stirred while 2M NaOH solution (1.03ml., 2.06mM) was added. Stirring was continued at room temperature for four hours after which tlc showed the saponification to be complete. The dioxane was removed under reduced pressure and the resulting oil was worked up in the same manner as for (163). The resulting crude product was recrystallised from CHCl_3 hexane mixtures to yield DppGly-Leu-MetOH (183) (826mg, 85%), m.p. 163–163.5°C; tlc $R_{f(B)}$ 0.26; (Found: C, 57.5; H, 6.6; N, 8.0. $\text{C}_{25}\text{H}_{34}\text{N}_3\text{O}_5\text{PS}$ requires C, 57.8; H, 6.6; N, 8.1%); $[\alpha]_{\text{D}}^{22}$ –24.3° (c 1 in MeOH); ν_{max} (CHBr_3 mull) 3600–2400 (CO_2H), 3380 3320 3195 (NH's), 1740 (acid C=O), 1660 (amide C=O's), 750 700 cm^{-1} (aromatic stretches); λ_{max} (MeOH) 205 (20,784), 224 (14,272), 260 (970), 266 (1,109), 274nm (831); δ_{H} (200MHz, CDCl_3) 0.9 (6H, dd, Leu δ -H's), 1.5 (3H, m, Leu β -CH₂ and γ -CH), 2.0 (5H, m, Met β -CH₂ and S-CH₃), 2.5 (2H, m, Met γ -CH₂), 3.4 (2H, m, Gly α -CH₂), 4.35 (2H, m, Leu and Met α -CH), 5.8 (1H, m, P-NH), 7.4–7.95 (10H, m, aromatic H's), 8.05 8.45 (2H, d's, Leu and Met NH's); δ_{C} (50MHz, CDCl_3) 14.5 (S-CH₃), 21.7

22.9 (Leu δ -C's), 24.0 (Leu γ -C), 29.8 30.7 (Met β and γ -C's), 41.4 (Gly α -C), 43.4 (Leu β -C), 50.9 51.1 (α -C's), 128.3-134.1 (aromatic C's), 169.6 171.9 172.8 (C=O's); δ_P (24MHz, $CDCl_3$) 28.2; m/z 520 320 258 219 (100%). HRMS 520.2035 ($[M+H]^+$); Amino acid analysis, Leu₁1.00, Met₁0.87, Gly₁1.00; hplc, R_t 18.4 mins (0-100%B):

N-Diphenylphosphinylglycylleucylmethionine amide (182)

DppGly-Leu-MetNH₂. (180) (1g, 1.87mM) was dissolved in a saturated solution of ammonia in methanol (5ml.) and stirred overnight when tlc showed the reaction to be complete. The solvent and excess ammonia were removed under reduced pressure and the resulting oil was triturated with petrol (40-60). The precipitated crude product was filtered and recrystallised from $CHCl_3$ hexane mixtures to give DppGly-Leu-MetNH₂ (688mg, 71%), m.p. 90-92°C; tlc, $R_{f(A)}$ 0.35; $[\alpha]_D^{22}$ -31.3° (c 1 in MeOH); ν_{max} ($CHBr_3$ mull) 3280 (NH), 1690 1650 1630 (amide C=O's), 750 700 cm^{-1} (aromatic stretches); λ_{max} (MeOH) 204 (24,479), 224 (15,351), 264 (1,660), 272nm (1,245); δ_H (200MHz, $CDCl_3$) 0.9 (6H, dd, δ -CH₃'s), 1.6 (3H, m, Leu β -CH₂ and γ -CH), 2.0 (5H, m, S-CH₃ and Met β -CH₂), 2.5 (2H, m, Met γ -CH₂), 3.65 (2H, m, Gly α -CH₂), 4.5 (3H, m, P-NH, Met and Leu α -CH's), 5.85 (1H, br.s, 1ry amide NH), 7.3 (1h, br.s, 1ry amide NH), 7.35-7.85 (10H, m, aromatic H's), 7.9 8.1 (2H, d's, NH's); δ_C (50MHz, $CDCl_3$) 15.2 (S-CH₃), 21.7 22.9 (Leu δ -C's), 24.9 (Leu γ -C), 30.4 30.9 (Met β and γ -C's), 41.1 (Gly α -C), 44.6 (Leu β -C), 52.4 53.1 (α -C's), 128.6-132.3 (aromatic C's), 171.2 172.5 173.9 (C=O's); m/z 519 371 343 258 230 201. HRMS 519.2195 ($[M+H]^+$); Amino acid analysis, Gly₁1.01, Leu₁0.99, Met₁0.93; hplc, R_t 13.4 mins (25-100%B):

N-Diphenylphosphinylphenylalanylglycylleucylmethionine methyl ester (184)

DppPhe-Gly-Leu-MetOMe. (180) (4.8g, 4.97mM) was dissolved in a minimum of methanol and a methanolic solution of hydrogen chloride (8.83ml., 20.3mM) was

added with stirring. The temperature was raised to 35–40°C and the reaction was stirred for thirty minutes after which it was shown to be complete by ^{31}P nmr and tlc. The solvent was removed and the residue was washed with ether several times and dried in a vacuum over phosphorus pentoxide. DppPheOH (2g, 5.47mM) was dissolved in dry DCM (10ml.) and cooled to –5°C with stirring. NMM (0.6ml., 5.47mM) and a solution of DppCl (1.29g, 5.47mM) were added dropwise and the reaction was left for fifteen minutes whereupon a solution of HCl.Gly–Leu–MetOMe (183) (4.97mM) in DMF (5ml.) and NMM (0.55ml., 4.97mM) were added. Stirring was continued for one hour at –5°C and one hour at ambient temperature. The solvent was removed under reduced pressure and the residual oil was worked up in the same fashion as for (94). The dried ethyl acetate solution was concentrated and the product was obtained by trituration with petrol (40–60) (2.4g, 71%), m.p. 205°C; tlc, $R_{f(A)}$ 0.52; (Found: C, 61.8; H, 6.6; N, 8.1. $\text{C}_{35}\text{H}_{45}\text{N}_4\text{O}_6\text{PS}$ requires C, 61.8; H, 6.7; N, 8.3%); $[\alpha]_D^{22}$ –62.4° (*c* 0.5 in MeOH); ν_{max} (CHBr₃ mull) 3300 (NH), 1740 (ester C=O), 1660 (amide C=O's), 750 cm^{-1} (aromatic stretches); λ_{max} (MeOH) 205 (28,866), 224 (15,522), 265 (2,179), 273nm (1,906); δ_{H} (360MHz, CDCl_3) 0.9 (6H, dd, Leu δ -CH₃'s), 1.75 (3H, m, Leu β -CH₂ and γ -CH), 1.95 (5H, m, S-CH₃ and Met β -CH₂), 2.5 (2H, dd, Met γ -CH₂), 3.1 (2H, m, Phe β -CH₂), 3.7 (4H, m, O-CH₃ and Phe α -CH), 3.8 (1H, m, P-NH), 4.0 (2H, m, Gly α -CH₂), 4.55 (1H, m, Leu α CH), 4.6 (1H, m, Met α -CH), 7.1–7.7 (17H, m, aromatic H's and NH 's), 8.2 (1H, d, Leu NH, $J_{\text{NH}-\alpha\text{CH}}$ 8.5Hz); Amino acid analysis, Phe₁1.01, Gly₁0.97, Leu₁1.02, Met₁0.55; hplc, R_t 20.5 mins (25–100%B):

N-Diphenylphosphinylphenylalanylglycylleucylmethionine (185)

DppPhe–Gly–Leu–MetOH. (184) (1g, 1.47mM) was suspended in peroxide free dioxane (10ml.) and 1M NaOH solution (1.6ml., 1.6mM) was added with stirring. Tlc showed the reaction to be complete after four hours. The solvent was

removed under reduced pressure and the residue was worked up in the same manner as for (164) to yield (187) after recrystallisation from ethyl acetate (754mg, 77%), m.p. 189–191°C; tlc, $R_{f(z)}$ 0.73, $R_{f(z)}$ 0.24; $[\alpha]_D^{22}$ –52.3° (c 1 in MeOH); ν_{\max} (CHBr₃ mull) 3280 (NH), 1730 (acid C=O), 1650 (amide C=O's), 750 700cm⁻¹ (aromatic stretches); δ_H (200MHz, d₆DMSO) 0.9 (6H, dd, Leu δ -CH₃'s), 1.65 (3H, m, Leu β -CH₂ and γ -CH), 2.0 (5h, m, S-CH₃ and Met β -CH₂), 2.45 (2H, dd, Met γ -CH₂), 2.95 (2H, m, Phe β -CH₂), 3.5 (1H, m, Phe α -CH), 3.8 (2H, m, Gly α -CH₂), 4.4 (2H, m, Leu and Met α CH's), 6.0 (1H, m, P-NH), 7.1–7.5 (15H, m, aromatic H's), 8.05 (1H, d, Leu or Met NH), 8.5 (1H, d, Leu or Met NH), 8.6 (1H, tr, Gly NH); δ_C (50MHz, d₆DMSO) 14.6 (S-CH₃), 21.5 23.0 (Leu δ -C's), 24.1 (Leu γ -C), 29.7 30.9 (Met β and γ -C's), 38.7 (d, Phe β -C, J_{p-C} 10.8Hz), 40.6 (Gly α -C), 42.5 (Leu β -C), 51.0 51.4 56.9 (α -C's), 126.4–138.4 (aromatic C's), 168.7 172.1 172.9 173.4 (C=O's); δ_P (24MHz, d₆DMSO) 21.7; m/z 667 201. HRMS 667.2719 ($[M+H]^+$); Amino acid analysis, Phe₁0.95, Gly₁1.00, Leu₁1.05, Met₁0.94; hplc, R_t 22.4 mins (0–100%B):

N-Diphenylphosphinylphenylalanylglycylleucylmethionine amide (186)

DppPhe-Gly-Leu-MetNH₂. (184) (681mg, 1mM) was dissolved in a saturated solution of methanol in ammonia (5ml.) and stirred overnight. Tlc showed the reaction to be complete and so the solvent and excess ammonia were removed under reduced pressure and the product was triturated out of solution. Recrystallisation from CHCl₃ hexane mixtures gave (188) as a white powder (473mg, 70%), m.p. 98–100°C; tlc, $R_{f(A)}$ 0.33; $[\alpha]_D^{22}$ –55.6° (c 0.5 in MeOH); ν_{\max} (CHBr₃) 3300 (NH), 1660 (amide C=O's), 750 730 700 (aromatic stretches); λ_{\max} (MeOH) 208 (34,470), 227 (16,260), 268 (1,463), 273nm (976); δ_H (360MHz, d₆DMSO) 0.85 (6H, dd, Leu δ -CH₃), 1.7 (3H, m, Leu β -CH₂ and γ -CH), 1.9 (3H, s, S-CH₃), 1.95 (2H, m, Met β -CH₂), 2.4 (2H, m, Met γ -CH₂), 2.9 (2H, m, Phe β -CH₂), 3.25 (3H, s, O-CH₃), 3.5 (1H, m, Phe α -CH), 3.8 (2H, m, Gly α -CH₂), 4.25

(1H, m, Met α -CH), 4.35 (1H, m, Leu α -CH), 6.0 (1H, m, P-NH), 7.1–7.35 (17H, m, aromatic H's and 1ry amide NH's), 7.7 (1H, d, Met NH, $J_{\text{NH}-\alpha\text{CH}}$ 8.3Hz), 8.56 (1H, d, Leu NH, $J_{\text{NH}-\alpha\text{CH}}$ 8.2Hz), 8.6 (1H, tr, Gly NH, $J_{\text{NH}-\alpha\text{CH}}$ 5.9Hz); δ_{P} (24MHz, d_6 DMSO) 21.5; m/z 666 405 320 201 (100%). HRMS 666.2879 ($[M+H]^+$); Amino acid analysis, Phe₁1.03, Gly₁0.93, Leu₁1.04, Met₁0.94; hplc, R_t 22.2 mins (0–100%B):

N-Diphenylphosphinylphenylalanylphenylalanylglycylleucylmethionine (188)

DppPhe-Phe-Gly-Leu-MetOMe. (184) (1g, 1.47mM) was dissolved in a minimum of methanol and warmed to 35–40°C when a solution of hydrogen chloride in methanol (4.2ml., 8.81mM) was added. The reaction was shown to be complete after thirty minutes by tlc and ^{31}P nmr. The solvent was removed under reduced pressure and the residue was triturated with dry ether yielding a white hygroscopic powder which was washed with more dry ether and dried over phosphorus pentoxide until needed. To a stirred solution of DppPheOH (592mg, 1.62mM) in DCM (5ml.) cooled to –5°C was added NMM (0.16ml., 1.62mM) and a solution of DppCl (383mg, 1.62mM) in dry DCM (1.9ml.) dropwise. The reaction was stirred for fifteen minutes after which a solution of HCl.Phe-Gly-Leu-MetOMe (187) (1.47mM) in DMF (5ml.) was added followed by NMM (0.15ml., 1.47mM). Stirring was continued for one hour at –5°C and one hour at room temperature. The solvent was removed and the residue was worked up using the same procedure as for (94) to yield DppPhe-Phe-Gly-Leu-MetOMe (188) after recrystallisation from ethyl acetate (584mg, 48%), m.p. 239–240°C; tlc, $R_{\text{f(A)}}$ 0.56; $[\alpha]_{\text{D}}^{22}$ –108.0° (c 0.5 in MeOH); ν_{max} (CHBr₃ mull) 3300 3210 (NH), 1740 (ester C=O), 1660 1630 (amide C=O's), 750 695cm^{–1} (aromatic stretches); λ_{max} (MeOH) 212 (55,310), 261 (3,974), 267 (4,637), 274nm (3,974); δ_{H} (360MHz, CDCl₃) 0.85 (6H, m, Leu δ -CH₃'s), 1.5 (3H, m, Leu β -CH₂ and γ -CH), 2.0 (2H, m, Met β -CH₂), 2.05 (3H, s, S-CH₃), 2.45 (2H, m,

Met β -CH₂), 3.0 (2H, m, Phe β -CH₂), 3.2 (2H, m, Phe β -CH₂), 3.5 (1H, m, Phe¹ α -CH), 3.7 (4H, m, O-CH₃ and P-NH), 4.0 (2H, m, Gly α -CH₂), 4.2 (1H, m, Phe² α -CH), 4.4 (2H, m, Leu and Met α -CH's), 6.9 (1H, d, Leu or Met NH), 7.0–7.5 (17H, m, aromatic H's and NH's); m/z 828 552 320 (100%) 201. HRMS 828.3560 ($[M+H]^+$); Amino acid analysis, Phe₂1.95, Gly₁1.01, Leu₁1.02, Met₁0.56; hplc, R_t 25.4 mins (0–100%B):

N-Diphenylphosphinylphenylalanylphenylalanylglycylleucylmethionine amide

(189) DppPhe-Phe-Gly-Leu-MetNH₂. (188) (66mg, 0.08mM) was dissolved in a saturated solution of ammonia in methanol (5ml.) and stirred for three days after which the reaction was shown to be complete by tlc. The solvent and excess ammonia were removed under reduced pressure and the product was triturated with hexane. The resulting off white powder was filtered and applied to a wet flash chromatography column eluted with 8% MeOH in CHCl₃ to yield the desired compound (41mg, 63%), ν_{\max} (CHBr₃ mull) 3300 (NH), 1650 (amide C=O's), 750 695cm⁻¹ (aromatic stretches); λ_{\max} (MeOH) 204 (33,495), 224 (13,333), 262 (1,138), 268 (1,303), 276nm (813); δ_H (360MHz, d₆DMSO) 0.8 (6H, dd, Leu δ -CH₃'s), 1.3 (1H, m, Leu γ -CH), 1.8 (2H, m, Leu β -CH₂), 2.05 (3H, s, S-CH₃), 2.2 (2H, m, Met β -CH₂), 2.6 (2H, m, Met γ -CH₂), 2.9 (2H, m, Phe β -CH₂), 3.2 (2H, m, Phe β -CH₂), 3.5 (2H, m, Phe β -CH₂), 3.8 (1H, m, P-NH), 3.9 (2H, m, Gly α -CH₂), 4.25 (1H, m, Phe α -CH), 4.45 (2H, m, Leu and Met α -H's), 5.25 5.7 (2H, br. s's, NH₂), 7.0–7.3 (23H, m, aromatic and NH's), 9.45 (1H, tr, Gly NH); m/z 813 552 320 201. HRMS 813.3563 ($[M+H]^+$); Amino acid analysis, Phe₂2.12, Gly₁0.97, Leu₁0.98, Met₁0.54; hplc, R_t 23.7 mins (0–100%B):

CHAPTER 5

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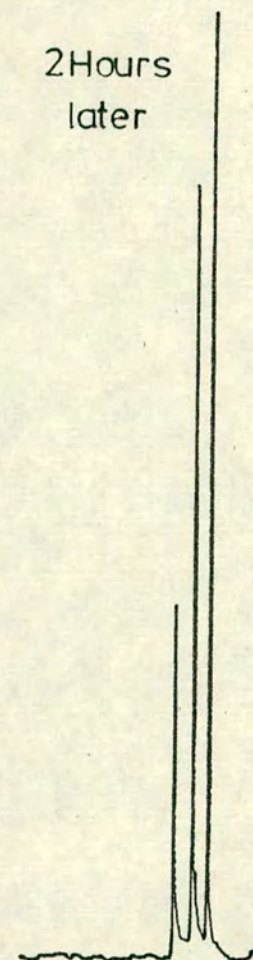
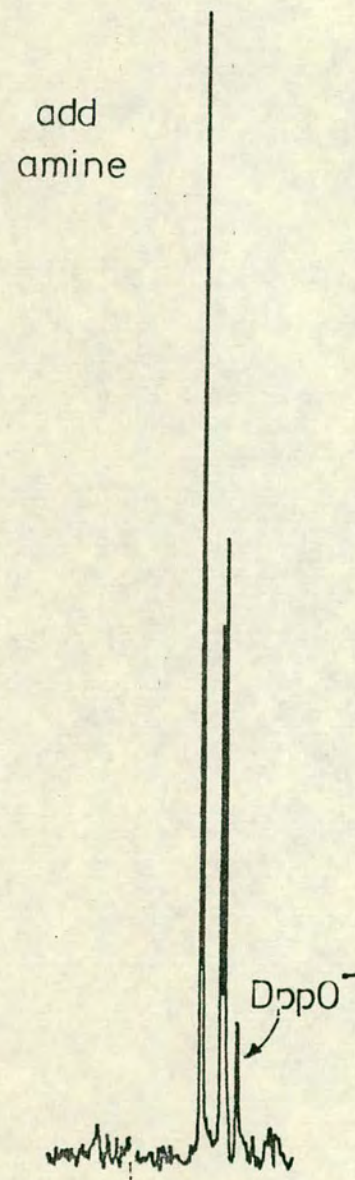
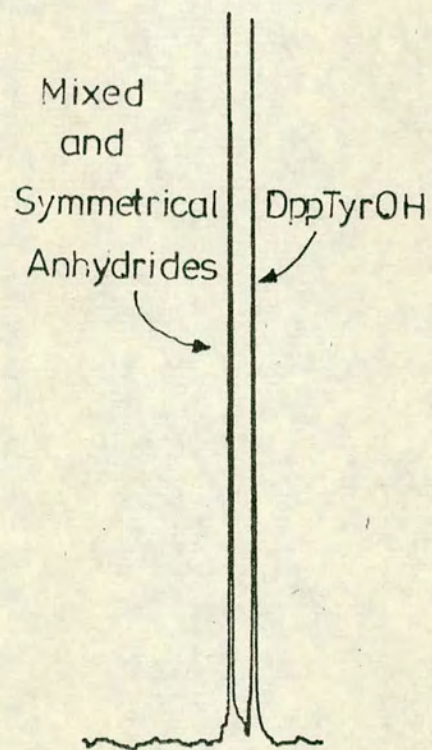
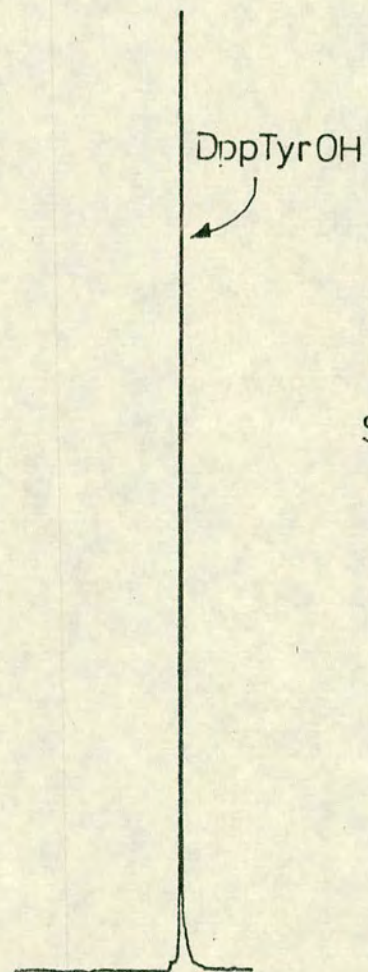
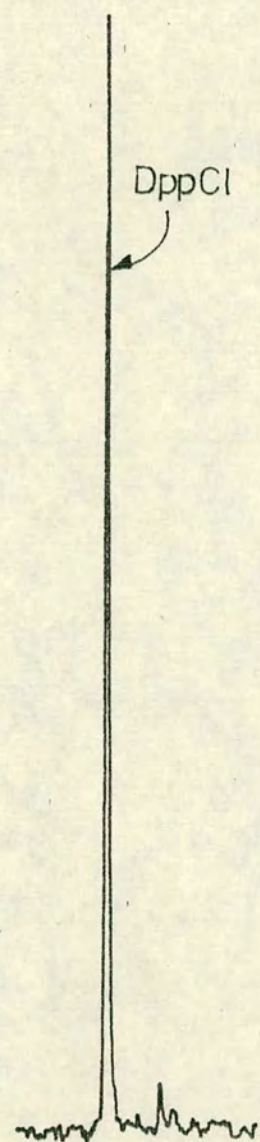
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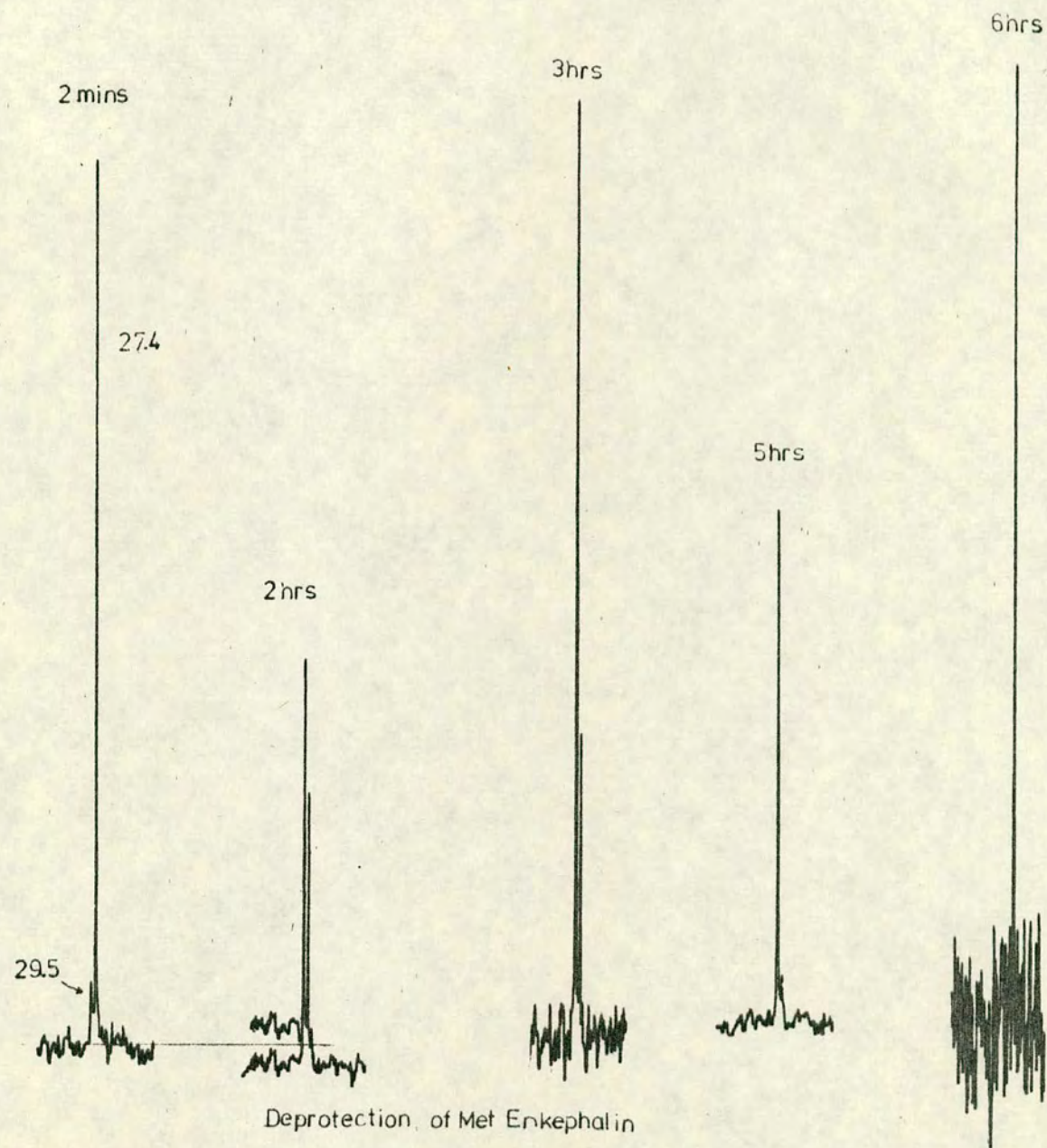
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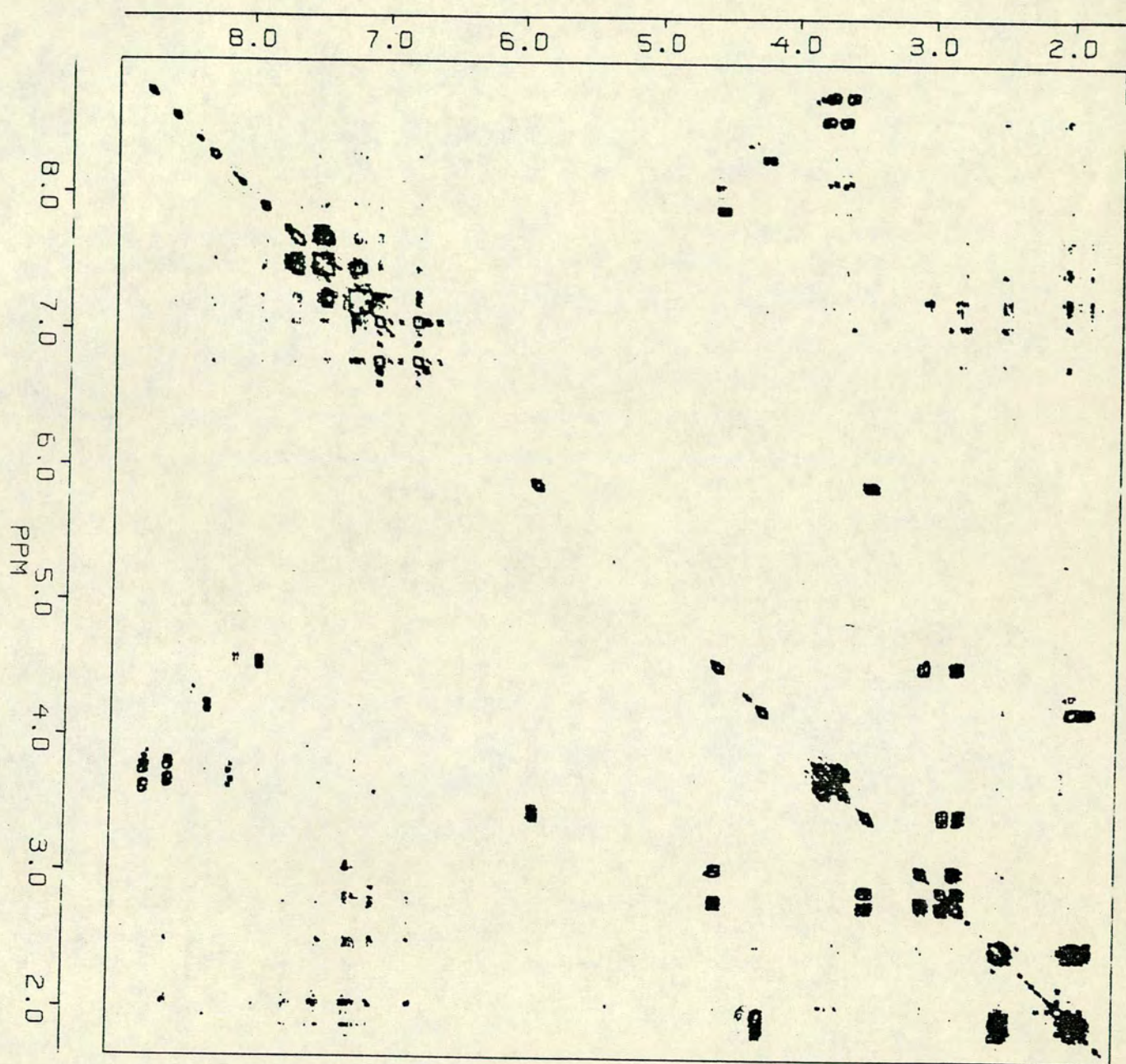
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Appendix II



2D cosy nmr of DppTyrGlyGlyPheMet